The Dissertation Committee for Gizelle Marin McCarthy Certifies that this is the approved version of the following dissertation:

Neuroimmune Signaling and Microglia in Chronic Ethanol Consumption

Committee:

R. Adron Harris, Supervisor

Roy Dayne Mayfield, Co-Supervisor

Jonathan Pierce-Shimomura

Vishwanath Iyer

Laura Nagy

Neuroimmune Signaling and Microglia in Chronic Ethanol Consumption

by

Gizelle Marin McCarthy, B.S.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May, 2017

Dedication

I would like to dedicate this dissertation to my mom, Joanne-Martin Robinson.

To my mom, the woman who made me the way I am, for better or for worse. To the woman told me not to cry over a "B" in 6th grade, when later my grandmother told me she had done the same thing. To the person who adored me despite what a challenging child I was. To the woman who loved to laugh. To the woman who had a vivid imagination and amazing artistic ability that made a 5-year-old me trace my books to be just like her. To the woman who taught me you don't give up, not even when you have cancer, not even when doctors tell you there is no hope. To the woman that I lost, but inspired me to fight. To the woman who made me realize that my inquisitive mind and unwillingness to accept "I don't know" meant I could make a difference. To the woman who inspired me to become a scientist and try to help others. To my mom.

Acknowledgements

I would like to acknowledge my mentor, R. Adron Harris, for his commitment to my scientific training. Through his guidance, I have learned so much about the scientific process, from devising a hypothesis and writing a grant to interpreting and presenting results. I would also like to acknowledge my co-mentor R. Dayne Mayfield for his support and mentorship. Dayne has provided me with invaluable guidance, particularly in the field of genomics which I had no experience with before entering the lab. With the help of Adron and Dayne, I have had a comprehensive graduate experience that has allowed me to grow scientifically.

I would like to thank my committee members for their help throughout the development of my dissertation project. With their feedback and advice, I have made the transition from proposing a project idea to defending my complete dissertation.

I would like to acknowledge the lab members for their help with my dissertation project. I would like to thank Sean Farris for all his mentorship and assistance with the genomic studies. I would also like to thank Olga Ponomareva, Elizabeth Osterndorff-Kahanek, and Courtney Bridges for their technical assistance with many aspects of my project. I would like to thank all past and current lab members for their scientific insight, contributions, and support.

Lastly, I would like to acknowledge my friends and my family. Without their support, I would not have made it through graduate school. I would like to specifically thank my husband Neil for having done every part of graduate school a few years before me and reassuring me that I will get through it.

Neuroimmune Signaling and Microglia in Chronic Ethanol Consumption

Gizelle Marin McCarthy, PhD

The University of Texas at Austin, 2017

Supervisor: R. Adron Harris

Co-Supervisor: Roy Dayne Mayfield

Alcohol use disorder (AUD) is a pervasive and debilitating condition characterized by the inability to stop consuming alcohol. Studies suggest that chronic alcohol use damages the prefrontal cortex (PFC), the brain region important for executive control, further driving alcohol consumption. Recent work has revealed that alcohol use alters immune signaling in the PFC, perturbing cortical function and increasing consumption. Although it is known that chronic alcohol use leads to neuroimmune changes in the brain, it remains unclear which specific innate immune signaling pathways are responsible. In addition, it is unknown how microglia, the immune cells of the brain, contribute to the ethanol-induced immune response *in vivo*. Thus, I sought to elucidate the specific immune pathways and microglial changes that occur in response to chronic voluntary ethanol.

Using a voluntary chronic drinking paradigm in mice, I evaluated gene expression changes in the Toll-like receptor (TLR) signaling pathways immediately following consumption and 24-hours after ethanol removal. I discovered that the primary TLR signaling pathway (the MyD88-dependent pathway) remained relatively unchanged, while

Tlr3 and components of its pathway (the TRIF-dependent pathway) were increased 24-hours after ethanol consumption in the PFC. I also looked at the nucleus accumbens (NAc) and amygdala (AMY) and found that the NAc showed similar changes to the PFC but weaker, while the AMY showed changes in the opposite direction. Furthermore, administration of a TRIF-pathway inhibitor decreased ethanol consumption, suggesting a role for this pathway in regulating drinking behavior.

Then, using isolated glial cells, I sought to determine which cell types TLR genes were localized in and which cell types showed changes following immune induction. I discovered that most TLR pathway genes are enriched and changed in microglia, however *Tlr3* is enriched and increased in astrocytes. These results suggest a potential role for TRIF-dependent signaling in astrocytes.

To further investigate the microglial changes in response to ethanol, I isolated the total homogenate and microglia from the PFC of mice that had undergone chronic voluntary ethanol consumption. I discovered that microglial changes were mostly undetected in the total homogenate and that microglial changes were associated with endosomal TLR signaling and TGF-β signaling. In addition, I investigated the microglial response to ethanol to the response to lipopolysaccharide (LPS), a TLR ligand that produces an immune response often compared to ethanol. Surprisingly, I found that microglia from the two treatments showed very different gene expression changes.

Together, these data suggest a role for endosomal TLR signaling, the TRIF-dependent pathway, and TGF-β in the ethanol-neuroimmune response. Future studies will aim to elucidate the mechanisms by which these signaling pathways change drinking, as well as the roles of the different cell types.

Table of Contents

List of Tables

List of Figures

Chapter 1: General Introduction and Significance

Despite many advances in science and medicine over the last century, brain-related pathologies remain poorly understood and therefore difficult to treat. Drug and alcohol addiction is one of these brain pathologies with a complex etiology, including genetic, psychological and environmental components. To develop improved treatments for addiction, there needs to be a more complete understanding of the biological processes that mediate the transition from use to dependence.

Recently, there has been an appreciation for the importance of immune signaling in the brain and how this signaling can contribute to many brain pathologies, including alcohol addiction. While it is known that neuroimmune signaling changes with chronic ethanol use and that this signaling can regulate consumption, it is unclear which signaling pathways mediate these changes and how different cell types in the brain contribute to this process.

1.I. Alcohol Use Disorder

Alcohol use disorder (AUD), characterized by heavy alcohol use and abuse, is a widespread societal problem. Affecting 6.2% of the adult population in the United States, AUDs contribute to an estimated 88,000 deaths per year making it the third leading cause of death (Center for Behavioral Health Statistics and Quality et al. n.d.; Centers for Disease Control and Prevention (CDC) n.d.). In addition to having devastating

consequences for the affected individual, AUDs also have detrimental effects on the rest of society. Every year in the US, alcohol-use is responsible for approximately 10,000 impaired-driving fatalities, 696,000 college sexual assaults, Fetal Alcohol Syndrome (FAS) in 2-7 births/1000, and more than 10% of children living with a parent with alcohol problems (National Center for Statistics and Analysis 2015; Substance Abuse and Mental Health Services Administration 2012; Hingson et al. 2005; May et al. 2009). AUDs also have a large economic impact costing the U.S. $249 billion per year (Bouchery et al. 2011).

Despite the prevalence and impact of AUDs, there are only three FDA-approved drugs and these treatments show limited success, particularly in reducing alcohol craving and relapse (Edwards et al. 2011). To develop more successful treatments, we need to better understand the genomic and molecular changes that contribute to the development of AUDs.

1.I.a. The Prefrontal Cortex and AUDs

Described in the Diagnostic and Statistical Manual of Mental Disorders 5, AUDs are characterized by tolerance, withdrawal, loss of control over consumption, and continued use despite negative consequences (American Psychiatric Association 2013). There are three main stages of alcohol use that contribute to the development of AUDs: the initiation of alcohol use driven by the rewarding properties, the continued use due to withdrawal symptoms and negative reinforcement, and the craving for alcohol that often drives relapse (Koob et al. 2014). Chronic alcohol use impacts many organs in the body,

however, the changes in the brain are most likely responsible for the transition from alcohol use to abuse and dependence. One brain region that is particularly sensitive to the effects of alcohol and important to the development of dependence is the prefrontal cortex (PFC).

The PFC is a region of the frontal cortex that is divided into three sub-regions: the medial PFC, the dorsolateral PFC, and the orbitofrontal cortex (Siddiqui et al. 2008). The PFC is responsible for executive function and has connections with sensory cortices, motor structures, and limbic regions that control memory and reward (E. K. Miller et al. 2002). There is controversy over how preserved the PFC is across mammalian species. While the PFC is different in size and structurally diverse across species, there is strong support from anatomical and functional data that rodents have a PFC, although less differentiated than in humans and non-human primates (Uylings et al. 2003).

Although the PFC is primarily involved in the craving of alcohol, it also contributes to the rewarding effects. The rewarding properties of alcohol that reinforce continued use come from activation of the mesocorticolimbic dopaminergic pathway, which includes the ventral tegmental area (VTA), the nucleus accumbens (NAc) and the PFC (Vengeliene et al. 2009). The PFC not only receives and integrates information from brain regions important for reward, but is responsible for key executive functions such as decision-making, planning, working memory, and impulse control (E. K. Miller et al. 2002). Many of these executive functions become impaired with chronic alcohol use and thus contribute to the loss of control and relapse associated with AUDs (Abernathy et al. 2010).

Alcohol-dependent human subjects show several structural changes in the PFC, including decreased neuronal density, reduced white matter volume and integrity, and reduced glial cell number (Oscar-Berman & Marinković 2007). In addition, fMRI scans have revealed many functional changes in the PFC of alcoholics, including reduced blood flow, event-related action potentials, and glucose metabolism (Oscar-Berman & Marinković 2007). Alcohol-dependent subjects also show decreased performance on behavioral tests, suggesting that these biological changes are driving the changes in executive control (Abernathy et al. 2010). For example, alcoholics show impairment on tests of memory, reversal learning, attention, decision making, and impulsivity. Many of these behavioral impairments seen in alcoholics are also seen in those with PFC lesions, further supporting the fact that the PFC regulates these behaviors (Bechara 2005). In addition, because the PFC interacts with limbic regions (e.g. amygdala and hippocampus), frontal cortical damage also causes changes in emotion and social behavior (Bechara 2005) (Oscar-Berman & Marinković 2007). Consistent with that idea, alcoholics display deficits in emotional perception and social function (Oscar-Berman & Marinković 2007).

Although it is difficult to causally show how cognitive dysfunction promotes continued alcohol use, impulse inhibition is thought to play an important role. While decision making is a complex process that requires attention, impulsivity requires little attention and focuses only on reward value without considering consequences (Crews & Boettiger 2009a). Alcoholics show deficits in inhibition response, and inhibition is further perturbed in response to alcohol cues (Noël et al. 2007). In addition, reduced

cortical activity in alcoholics during decision making correlates with choice of immediate over delayed rewards, while naltrexone (FDA approved drug for AUDs), increases activity and improves decision making (Boettiger et al. 2007; Boettiger et al. 2009). Furthermore, disinhibition and antisocial personality are highly correlated with early-onset alcoholism and chronic alcohol use and dependence (Oscar-Berman & Marinković 2007). Consistent with the idea that cortical dysfunction promotes consumption, the degree of cortical dysfunction in abstinent alcoholics is predictive of the probability of relapse {Burnett:2016ka}. There is also evidence that frontal cortical dysfunction is genetic, and thus not only promotes consumption in dependent subjects, but is a factor in the initiation of alcohol use and abuse.

Although there is some understanding of how alcohol changes the PFC and therefore drives addiction, a better understanding of the molecular processes that cause cortical dysfunction is needed to develop drugs that prevent relapse.

1.I.b. Mouse models of AUD

Although human alcoholic data is informative and necessary, to better study alcohol in a controlled setting, several rodent paradigms have been developed to model different aspects of AUDs. Although human studies of alcoholics have provided insight into structural and molecular abnormalities in the PFC, they make it impossible to determine whether these aberrations predisposed the individual to alcohol use or were the consequence of it. Voluntary ethanol consumption models are advantageous because they can be used to study both cause and effect. With a voluntary consumption paradigm,

genetically identical mice can be allowed to drink ethanol and changes can be measured compared to a control group. Once molecular changes are identified, the same strain of mice can be manipulated to determine how a particular molecular change can alter ethanol consumption.

There are several types of rodent voluntary ethanol consumption paradigms which differ in ethanol percentage, access to water, and frequency and duration of ethanol access. In C57BL/6J mice, 24-hour intermittent access to both alcohol and water leads to an escalation in ethanol consumption and higher levels of consumption than continuous access (Crabbe et al. 2012). Every-other-day ethanol access is thought to model human consumption better than continuous access because of the escalation in drinking, which is seen in individuals with AUDs. This escalation is attributed to increases in binge like drinking and anticipation of the reinstatement of alcohol (Melendez 2011). Consequently, voluntary every-other-day 2-bottle choice (EOD-2BC) paradigms are used to measure changes that result from continuous bouts of ethanol consumption and are useful in determining how any biological or environmental manipulations may alter consumption.

1.II. Alcohol and Neuroimmune Signaling

The transition from initial alcohol use to dependence involves several genomic and molecular changes. Recent studies suggest that neuroimmune signaling is an important mediator of this shift, particularly in the PFC. It is hypothesized that chronic ethanol consumption leads to neuroimmune changes that impair cortical function and promote dependence.

1.II.i. Defining "neuroinflammation"

I would like the preface this section with the issue of defining "neuroinflammation". Neuroinflammation is simply defined as inflammation of the nervous tissue, while inflammation is defined as the reaction of living tissues to injury, although neither of these definitions are particularly informative (Streit et al. 2004). Other definitions of neuroinflammation include processes such as: the release of proinflammatory mediators, activation of glial cells, damage to the blood brain barrier, leukocyte infiltration, and contribution to neurodegeneration (O'Callaghan et al. 2008; Streit et al. 2004). The exact definition of neuroinflammation remains controversial, as do the criteria for how pronounced the neuroimmune response must be to qualify as "neuroinflammation". Although it remains controversial whether the neuroimmune changes produced by stimuli such as alcohol meet the criteria of neuroinflammation, for lack of a better noun, I will use term inflammation/neuroinflammation to describe the impact of perturbed neuroimmune signaling throughout this dissertation.

1.II.a. Neuroimmune signaling in the brain

It was previously thought that due to the blood-brain barrier the brain was an immune privileged organ. It is now widely accepted that the brain has its own specialized immune response, often referred to as neuroimmune signaling (Dantzer et al. 2008). Recent work suggests that neuroimmune signaling plays an important role in brain pathologies, including alcohol addiction (Ransohoff 2016b; Vetreno & Crews 2014; Cherry et al. 2014; Hayley 2014). Neuroimmune signaling encompasses both innate and adaptive immunity, however, the focus in alcohol research is largely the innate immune response (Vetreno & Crews 2014). Innate immunity is a nonspecific response to pathogens that involves the recruitment of immune cells and the production of cytokines, small immunomodulatory

agents involved in autocrine, paracrine, and endocrine signaling. Immune cells in the body identify pathogens and endogenous ligands through pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs) or damage-associate molecular patterns (DAMPs). In response to PAMPs or DAMPs, immune cells activate immune related signaling pathways and transcription factors. These responses lead to the release of cytokines and reactive oxygen species, which then act on surrounding cells and amplify the immune response (Mogensen 2009).

The peripheral immune response can activate the neuroimmune response through cytokine-activation of afferent nerves, cytokine diffusion from the blood to the brain, and cytokine transport across the blood-brain barrier (Dantzer et al. 2008). Peripheral immune signaling then leads to activation of the resident immune cell of the CNS, microglia. Activated microglia secrete cytokines that serve to amplify the immune response by signaling to other microglia as well as astrocytes and neurons (Vetreno & Crews 2014). In addition to microglia, other CNS cells, including astrocytes and neurons, also participate in the immune response and amplification (*discussed in more detail in 1.III.a.iii. Microglial interaction with other cell types, 1.IV.c. Localization of TLR signaling*). The immune response can be both pro- and anti-inflammatory and can be beneficial by killing pathogens or repairing damage. However, the pro- and anti- inflammatory balance is important and the immune response can also cause damage in response to chronic inflammation, such as the inflammation resulting from long-term alcohol abuse (Vetreno & Crews 2014).

1.II.b. Alcohol's impact on immune signaling

Alcohol's effects on immune signaling throughout the body and peripheral immune system work in concert with neuroimmune signaling. Alcohol alters peripheral immune

signaling by increasing intestinal permeability (*more detail in 1.V.a.i. Indirect effects via peripheral immune signaling*). Because the intestines are colonized by large amounts of bacteria, increased intestinal permeability can cause bacteria to leak out of the intestines into the blood stream. Components of bacterial molecules, such as the endotoxin lipopolysaccharide (LPS), can act as PAMPs and lead to immune activation and cytokine production. Peripheral immune activation then causes an immune response in other peripheral organs (e.g. liver) and in the CNS (Leclercq et al. 2017). The peripheral and CNS immune response differ in latency and duration as the neuroimmune response takes longer to occur but is sustained longer (Qin et al. 2008). In addition, acute and chronic ethanol consumption differ in their effects on the immune system. Acute alcohol exposure suppresses the peripheral immune response while chronic ethanol exposure increases neuroinflammation (Vetreno & Crews 2014).

Although immune signaling in response to ethanol has been studied in the periphery for many years, particularly with regards to alcoholic liver disease, there is a growing appreciation for alcohol's effects on neuroimmune signaling. The link between alcohol and immune signaling in the brain was originally discovered in gene expression studies in the human alcohol cortex (Lewohl, L. Wang, Miles, Zhang, Dodd & Harris 2000a). Since then, several *in vitro* culture, rodent, and human postmortem alcoholic studies provide evidence for ethanol-induced neuroimmune signaling.

In vitro ethanol exposure causes increased cytokine expression in macrophage cultures, as well as primary cultures from the CNS immune cells microglia and astrocytes. These cytokines include tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β), which are typically pro-inflammatory and amplify the immune response (Fernandez-Lizarbe et al. 2009; Alfonso-Loeches et al. 2010; Fernandez-Lizarbe et al. 2008). Additionally, ethanol-exposed cultures show increased expression of enzymes involved in

the inflammatory response, inducible nitrous oxide synthase (iNOS) and cyclooxygenase 2 (COX-2), and the immune transcription factor nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) (Fernandez-Lizarbe et al. 2009; Blanco et al. 2008). Rat brain slice cultures also show several immune changes in response to ethanol, including increased NF-κB DNA binding and expression of cytokines, endogenous DAMPS, and iNOS (Zou & Crews 2010; Crews et al. 2013).

Binge ethanol exposure in rodent models leads to increased expression of the Toll-like receptors (TLRs), a category of PPR, and their endogenous DAMP high mobility group box 1 (HMGB1) (Crews et al. 2013). Binge ethanol also leads to increased cytokine expression (MCP-1/CCL2, IL-6) (Kane et al. 2013), increased COX-2 expression, increased NF-κB binding, and glial cell activation (Crews et al. 2006; Crews et al. 2013). Similarly, chronic ethanol exposure in rodents leads to increased TLR and endogenous ligand expression, increased cytokine expression (MCP-1, IL-1β, TNF-α), increased COX-2 and iNOS expression, and glial activation (Whitman et al. 2013; Lippai et al. 2013; Alfonso-Loeches et al. 2010). Many of these immune changes were found in the cortex, highlighting the importance of immune signaling in this brain region.

Consistent with evidence *in vitro* and in rodent models of alcohol consumption, several immune changes are found in post-mortem alcoholic brains. These changes include microglial activation, increased expression of TLRs and HMGB1, and increased cytokine expression (Vetreno et al. 2013; He & Crews 2008; Crews et al. 2013). Together these studies suggest that alcohol impacts neuroimmune signaling with respect to PAMPs/DAMPs, PRRs, cytokine production, and glial activation.

1.II.c. Immune signaling and ethanol-induced damage

One of the consequences of ethanol-induced immune signaling is cell or organ damage and dysfunction. This ethanol-induced inflammatory damage is well established in the liver where it contributes to the development of alcoholic liver disease (Szabo et al. 2011; Szabo & Lippai 2014). Neuroimmune signaling is also involved in the ethanol-induced brain damage observed in alcoholics. Human alcoholic brains show damage, including reduced gray and white-matter volumes, neuronal loss, and decreased glial counts and size, and this damage is particularly apparent in the frontal cortex (Abernathy et al. 2010; Vetreno & Crews 2014). Recent studies suggest that ethanol-induced neuroinflammation contributes to neuronal damage and neurodegeneration (M. A. Pascual et al. 2011; Zou & Crews 2010; Qin et al. 2008).

Chronic alcohol induces microglial and astroglial activation, production of cytokines and reactive oxygen species, cell death, and cognitive dysfunction in mice (Alfonso-Loeches et al. 2010; Blanco et al. 2005; Fernandez-Lizarbe et al. 2009; M. A. Pascual et al. 2011). Studies reveal that the PPR TLR4 is essential for these ethanol-induced neurotoxic and behavioral deficits, further supporting the idea that neuroimmune signaling contributes to the brain damage caused by alcohol consumption (M. A. Pascual et al. 2011; Alfonso-Loeches et al. 2010). There are several mechanisms by which inflammation leads to damage. Studies suggest that ethanol causes a shift in transcription, decreasing binding of cyclic AMP response element-binding protein (CREB) transcription factors which promote neuronal survival, while increasing NF-κB binding, which transcribes cytokines and promotes oxidative stress (Crews & Kim Nixon 2009). The cytokine TNFα, in conjunction with reactive oxygen species, can cause apoptosis (Hsu et al. 1996; Kamata et al. 2005). Neuroimmune signaling also causes a decrease in astrocytic glutamate transport causing hyperexcitability, and increased extracellular glutamate,

leading to neurotoxicity (Zou & Crews 2005a; Vetreno & Crews 2014). In addition, ethanol reduces cortical and hippocampal neurogenesis (formation of new neurons) by inhibiting neural stem cell proliferation and survival, and this reduction is reversed by blocking the PPR interleukin 1 beta (*Il1b)* receptor or inhibiting NF-κB (Zou & Crews 2010; Crews et al. 2006; Zou & Crews 2012; Kimberly Nixon & Crews 2002; Crews & Kim Nixon 2009).

Pharmacological studies further support the idea that neuroimmune signaling potentiates ethanol-induced damage. Adolescent rats undergoing binge drinking show increased COX-2 and iNOS, cell death, and behavioral deficits, which are prevented by the COX-2 inhibitor Indomethacin (M. Pascual et al. 2007). Supporting the hypothesis that NF-κB induced oxidative stress promotes damage, the anti-oxidant butylated hydroxytoluene also blocked COX-2 induction, neuronal death, and increased neurogenesis (Crews et al. 2006). In addition, Rolipram, which increases CREB-DNA binding, reduced ethanol- induced neurodegeneration (Crews & Kim Nixon 2009). Minocycline and naltrexone, drugs that are known to block microglial activation, also reduced neuroinflammation and cell death in response to ethanol (Qin & Crews 2012). These studies highlight the effects of neuroimmune signaling on CNS damage and suggest that drugs targeting inflammation may be beneficial to alcoholics.

1.II.d. Immune signaling regulates ethanol consumption

In addition to contributing to neuronal damage, neuroimmune signaling can regulate ethanol consumption. The first hint of a correlation between immune genes and ethanol came from human post-mortem genomic studies, however, those data don't show whether consumption increased gene expression or gene expression increased consumption (Jianwen Liu et al. 2006). Consequently, genomic studies using abstinent animals from

rodent lines that are known to consume high or low amounts of alcohol, revealed differences in immune gene expression (Mulligan et al. 2006). These data then suggested a causal role for immune genes in regulating ethanol intake. Consistent with differences in rodent lines, polymorphisms for immune genes are associated with risk of developing AUDs (Cui et al. 2014).

Several studies have shown that systemic increases in immune signaling promote ethanol consumption while deletion of immune genes reduce consumption. Injection of the bacterial endotoxin LPS leads to persistent increases in ethanol consumption (Blednov, Benavidez, et al. 2011), while knockout mice for immune genes show decreased ethanol consumption. Chemokines are a family of small cytokines that induce chemotaxis, and transgenic mice with deletions of C-C motif chemokine ligands 2/3 (*Ccl2, Ccl3*) and the C-C motif chemokine receptor 2 (*Ccr2*) genes show reduced ethanol preference and consumption (BLEDNOV et al. 2005). Several immune-related genes previously linked to alcohol consumption in gene expression studies were also validated *in vivo*. Knockout mice for several other immune genes, beta-2 microglobulin (*B2m*), cluster of differentiation 14 (*Cd14*), interleukin-1 receptor antagonist (*Il1rn*), interleukin 6 (*Il6*), cathepsin S (*Ctss*), and cathepsin F (*Ctsf*), all showed decreased ethanol consumption and preference in a 24-hour 2-bottle choice test (Blednov, Ponomarev, et al. 2011).

Additionally, CNS specific manipulations of immune genes can alter ethanol consumption, highlighting the role of immune signaling in the brain. Knockdown of IKKβ, a kinase involved in activation of NF-κB, in the nucleus accumbens or central amygdala, reduced voluntary ethanol consumption in mice (Truitt et al. 2016). In addition, knockdown of the PPR TLR4 in the amygdala of alcohol-preferring rats reduces binge drinking (Juan Liu et al. 2011). Global knockout of TLR4 or knockdown in the ventral pallidum did not change ethanol consumption, highlighting the complexity of neuroimmune signaling and

the importance of studying the response in specific brain regions (Juan Liu et al. 2011; Harris et al. 2017).

Pharmacological studies also provide evidence for immune regulation of ethanol consumption. Minocycline, an antibiotic with immune-modulatory properties, significantly reduced voluntary ethanol consumption in mice (Agrawal, Hewetson, C. M. George, Syapin & Bergeson 2011a). In addition, two inhibitors of IKKβ, TPCA-1 and sulfasalazine, decrease ethanol intake and preference in mice (Truitt et al. 2016). These results not only support the involvement of neuroimmune signaling in the regulation of consumption, but suggest that drugs targeting immune signaling may be promising for the treatment of AUDs.

1.III. MICROGLIA

In the adult brain microglia are confined to the CNS and make up approximately 5-10% of cells, however, microglia have a distinct lineage compared to other brain cell types (Lawson et al. 1990). While neurons, astrocytes and oligodendrocytes are derived from neural stem cells, microglia are derived from yolk-sac hematopoietic stem cells (Ransohoff & Cardona 2010). Myeloid progenitor cells enter the neural tube at E8.5 in rodents and differentiate into microglia (Salter & Beggs 2014). The microglial population in the adult CNS is a finely tuned balance of proliferation and apoptosis, and microglia are mostly maintained through local self-renewal instead of repopulation from bone marrow macrophages (Askew et al. 2017). Compared to other tissue macrophages, microglia are uniquely regulated and have a distinct genomic signature (Salter & Beggs 2014). Consequently, microglia are specialized cells that have important immune and CNS

functions which vary depending on whether the brain is in a healthy or pathological state (**Fig. 1.1**).

1.III.a. Microglial Function

1.III.a.i Microglial Function under physiological conditions

Under physiological conditions, microglia are in a "ramified state" during which they have a small cell body with long processes and are continuously surveying the entire brain. Although this surveillance is in part to detect any disease or damage, it is also to interact with other CNS cells, including neural stem cells and differentiated neurons (Salter & Beggs 2014).

During postnatal development, microglia are responsible for regulating the number of neuronal precursors and neurons. Microglia regulate neuron number by inducing apoptosis and phagocytosing dispensable neurons, controlling differentiation of precursor cells, and providing trophic support to necessary neurons (Salter & Beggs 2014). Additionally, microglia play an important role in synaptic pruning during development, a process important for refining neuronal connectivity. Synaptic pruning is dependent on synaptic activity and microglia respond to neuronal activity and signals, eliminating inactive synapses (Salter & Beggs 2014). Microglia are also involved in maturation of the excitatory synapses by altering the composition of NMDA receptor subunits and the ratio of AMPA to NMDA receptors (Salter & Beggs 2014).

Aside from their role in development, microglia also play an important role in the healthy adult brain. Learning and memory involve activity-dependent synaptic plasticity, through the process of long-term potentiation (LTP) or long-term depression (LTD). Microglia are critical for activity-dependent synaptic plasticity, as evidenced by deficits in

LTP and glutamate recycling in mice with dysfunction microglia (CNS-TGFβ1-deficient mice) (Koeglsperger, Li, Brenneis, Saulnier, Mayo, Carrier, Selkoe & Weiner 2013a). Microglia show increased contact with highly active neurons, and this contact seems to lead to a reduction in spontaneous and evoked calcium activity (Y. Li et al. 2012). Thus, it appears that microglia monitor the synaptic function of neurons they are in contact with, and can alter neuronal activity in response. Additionally, microglial depletion in adult mice results in altered spine dynamics and deficits in motor learning (Parkhurst et al. 2013).

In addition, microglia continue to regulate neurogenesis in the adult CNS (Luo & S.-D. Chen 2012). Microglia not only participate in the "pruning process" by removing apoptotic cells, but can also influence proliferation, differentiation and survival or progenitor cells (Gemma & Bachstetter 2013). Progenitor cells grown in conditioned microglial media show a higher proportion of neuronal cells, while neurons grown in conditioned media have increased survival. Studies suggest that microglia secrete growth factors, including insulin-like growth factor (IGF) and brain-derived neurotrophic factor (BDNF), that promote neurogenesis and survival. Additional studies support the idea that microglia beneficially impact neurogenesis, suggesting that microglial dysfunction may be involved in the decreased neurogenesis seen in aged mice (Luo & S.-D. Chen 2012).

Together, new data support an important role for microglia in normal brain development and synaptic plasticity and future studies will further elucidate these processes. These results highlight how alterations in microglial activity can impact brain function, even in the absence of inflammation.

1.III.a.ii Microglial Function under pathological conditions

Despite their important roles in the healthy brain, microglia are primarily known for being the resident immune cells of the brain. Microglia are constantly surveying the brain and if they encounter PAMPS or DAMPs, they become 'activated'. Activated microglia change their morphology from elongated processes to a more amoeboid shape with a larger cell body and shorter processes and can become phagocytic (Ransohoff & Cardona 2010). Activated microglia adopt different phenotypes, but microglial immune response usually results in a shift from homeostatic function to immune-related functions.

Although controversial, it is believed that activated microglia can enter either a classical-activation state (M1-like), an alternative activation state (M2-like), or acquired deactivation state (M0) (**Fig. 1.2**) (Luo & S.-D. Chen 2012; Ransohoff 2016a). The M1 and M2 states are based on differences in gene expression originally identified in peripheral macrophages, but have some support in CNS studies (Ransohoff & Cardona 2010). The M0 or acquired deactivation state is recently identified and is like the M2 state. The phenotypic distinction between M0 and M2 is still being elucidated and the M0 state often has different names, is considered part of M2 (M2c), or is defined as the homeostatic state. It is worth noting that many studies investigating microglial activation states use *in vitro* cultures, which differ substantially from microglia *in vivo* (Schmid et al. 2009; Ransohoff 2016a). New techniques are emerging to better capture the *in vivo* microglial response and help to further characterize microglial states. It is mostly agreed upon that microglia can exhibit pro-inflammatory or anti-inflammatory properties, but that these properties exist in a continuum as opposed to distinct states and that these responses may be different than those observed in macrophages or *in vitro* microglia.

Classically activated microglia are characterized by the inducing agent IFN-γ or LPS and the production of pro-inflammatory cytokines, reactive oxygen and nitrogen

species, and antigen-presenting molecules (Cherry et al. 2014; Colton 2009). Specific markers of M1 activation include IL-1β, IL-6, TNF-α, CCL2, ROS, and NO. The purpose of classical activation is to mount an inflammatory response within the CNS and defend against bacterial and viral infections (Nakagawa & Chiba 2014). However, the pro-inflammatory response mounted to destroy pathogens, can also be neurotoxic (Y. Tang & Le 2016). Furthermore, the PRRs responsible for recognizing pathogens are also capable of detecting endogenous molecules (e.g. miRNAs, misfolded proteins, damaged cells and organelles), leading to an unnecessary pro-inflammatory response (Sochocka et al. 2016). Chronic activation of microglia in a pro-inflammatory/M1-like state can lead to mitochondrial dysfunction, impaired neuronal function, apoptosis, and impairment of the blood brain barrier (Sochocka et al. 2016).

Conversely, alternatively activated or acquired deactivated microglia release anti-inflammatory cytokines and serve to suppress inflammation and promote repair (Luo & S.-D. Chen 2012). The major difference between the M2 and M0 states is the ligand that stimulates them, with M2 being induced in response to IL-4 and IL-13 and M0 being induced in response to TGF-β, IL-10, or apoptosis (Luo & S.-D. Chen 2012; Colton 2009; Y. Tang & Le 2016). IL-4, IL-13, and IL-10 are well known anti-inflammatory cytokines, while transforming growth factor beta (TGF-β) is an anti-inflammatory cytokine and growth factor that has many functions. In addition to influencing the immune response, TGF-β has important roles in development, cell-cycle, apoptosis, migration, angiogenesis, and extracellular matrix (ECM) remodeling (X. Guo & X.-F. Wang 2009).

Markers of M0/M2 activation include the surface markers Arg-1, Ym1, CD36, CD163, and IL-10. (Nakagawa & Chiba 2014). M2 activation can lead to the production of TGF-β and promote M0 activity, while M0 microglia can upregulate markers that enhance M2 activity (Y. Tang & Le 2016). Production of anti-inflammatory cytokines by

M2 microglia can also downregulate the pro-inflammatory response in M1 microglia. Interestingly, there is also evidence that pro-inflammatory cytokines (CCL2, IL-6) produced by M1 microglia may induce M2 polarization to begin the repair process (Nakagawa & Chiba 2014). M2 microglial activation increases expression of genes involved in tissue and ECM repair, promotes phagocytosis of cell debris, and releases neurotrophic factors to support neuronal survival (Y. Tang & Le 2016).

Despite the discrete classifications, activation states exist as a spectrum and it is unclear whether different activation states can exist simultaneously (Nakagawa & Chiba 2014; Y. Tang & Le 2016). For the CNS to function correctly, a balance between pro- and anti-inflammatory responses is important. Disruption of this balance is what leads to chronic neuroinflammation and damage (Luo & S.-D. Chen 2012; Carniglia et al. 2017; Cherry et al. 2014). Although an M1-response seems detrimental, while an M2-response seems beneficial, the impact of these responses in CNS pathology is context dependent. For example, an overactive pro-inflammatory response (attributed to M1-microglia) is thought to contribute to Schizophrenia and depression (Nakagawa & Chiba 2014). In contrast, dysfunctional M2 response and TGF-β signaling is implicated in aging and Alzheimer's disease (Bernhardi et al. 2015). Furthermore, timing of microglial responses are important, and there is evidence that in certain diseases (e.g. experimental allergic encephalomyelitis) a specific response might be beneficial at one time point but detrimental at another (Luo & S.-D. Chen 2012).

1.III.a.iii. Microglial interaction with other cell types

Microglia not only regulate neuronal processes, but are also strongly regulated by neurons. When healthy, neurons send inhibitory signals to microglia preventing activation

(Luo & S.-D. Chen 2012). Interactions between neurons and microglia are often mediated by interactions between ligands that are expressed on neurons and their receptors expressed on microglia, such as CD200-CD200R, CD22-CD45, and CX_3CL1-CX_3CR1(Ransohoff & Cardona 2010). In addition to direct interactions, neurons also regulate microglia through electrical activity and soluble factors. In response to neuronal damage, these inhibitory signals get disrupted, leading to microglial activation.

Microglia also continuously interact with astrocytes within the CNS. Although microglia-astrocyte interactions during homeostasis remain unclear, the two cell types have many interactions during inflammation. Activated microglia induce A1 (neurotoxic) astrocytes by secreting Il-1α, TNF, and C1q (Liddelow et al. 2017). Activated astrocytes, in turn, reduce glutamate transport, leading to increased levels of extracellular glutamate and ultimately excitoxicity (Bal-Price & Brown 2001). In response to activation, astrocytes can secrete cytokines and further activate microglia through calcium waves or they can suppress cytokine production and decrease microglial toxicity (Luo & S.-D. Chen 2012). Microglial signals can also alter astrocyte proliferation, glutamate activity, and survival (Luo & S.-D. Chen 2012).

Oligodendrocytes have a less appreciated role in the neuroimmune response, but they regulate myelination which is impacted in neuroinflammatory conditions like Multiple Sclerosis (MS). Microglia are known to regulate survival, differentiation, and proliferation of oligodendrocyte progenitor cells (OPCs) during development (Miron 2017). Microglia also play an important role in re-myelination, by phagocytosing myelin debris to make way for OPCs (Miron 2017). In addition, activated microglia directly promote OPC proliferation, differentiation, and remyelination.

Although microglial interactions are confined to the CNS in healthy brain, disruption of the blood-brain barrier leads to infiltration of other immune cell types that

can interact with microglia. Activated microglia can function as antigen presenting cells that recruit T-cells from the periphery. Recruited T-cells, in turn, can directly or indirectly modulate the microglial response (Luo & S.-D. Chen 2012).

1.III.b. Microglial changes with ethanol consumption

1.III.b.i. Changes in morphology and marker expression

Microglial under normal conditions are considered "ramified" and have a small cell body and several long processes that are continuously monitoring the parenchyma (Kovács 2017). However, in response to danger or damage, microglia become activated and begin to mount an immune response, characterized by a bushy morphology with a larger cell body and smaller processes. Fully activated and phagocytic microglia take on an amoeboid appearance (He & Crews 2008). In addition, expression of microglial markers is upregulated in response to microglial activation (Imai & Kohsaka 2002).

Studies have shown that both LPS and ethanol can change expression of microglial markers and microglial morphology in several models. In cultured primary microglia, ethanol treatment led to a more activated morphology, characterized by a more bushy and amoeboid appearance (Fernandez-Lizarbe et al. 2009). In ethanol pre-treated mice injected with LPS, microglia showed increased size and changes in morphology consistent with microglial activation (Qin et al. 2008). Chronic ethanol consumption in mice leads to increased levels of microglial markers (Cd11b and Iba1) in the cortex and cerebellum while binge ethanol results in microglial activation indicated by an amoeboid morphology and thicker processes (Alfonso-Loeches et al. 2010; Lippai et al. 2013; Crews et al. 2006). Human postmortem data also supports the idea that alcohol exposure leads to microglial activation. In human alcoholic postmortem brains, the cingulate cortex shows increased

microglial marker (Iba-1 and $GluT_5$) immunoreactivity compared to control brains (He & Crews 2008).

Interestingly, imaging studies using the microglial activation marker 18-kDa translocator protein (TSPO) show decreases with chronic alcohol. Positron emission tomography revealed 10% lower TSPO levels in the striatum and hippocampus of alcohol-dependent subjects compared to healthy controls (Hillmer et al. 2017). An additional study found that hippocampal TSPO levels correlated with verbal memory performance in healthy controls but not in recently abstinent alcohol dependent subjects (Kalk et al. 2017). These results highlight the complexity of the neuroimmune response to ethanol. Although TSPO is primarily expressed in microglia and reactive astrocytes, it is also found in some neurons and has high expression in endothelial cells (Rupprecht et al. 2010; Y. Zhang et al. 2014). Furthermore, decreased TSPO expression in neurons regulates ethanol-responsive behaviors in Drosophila (Lin et al. 2015). It is possible that TSPO is changing differently in different cell types. In addition, it is possible that TSPO function is changing despite microglial activation or that it is labeling a different subtype of microglia than other markers. Consistent with the complexity of these measurements, a recent study fund that down-regulation of TSPO does not change cytokine induction in a microglial cell line (Dou et al. 2014). Future work elucidating the role of TSPO in microglia will be necessary to reconcile contrasting results about microglial activation.

.

1.III.b.ii. Microglial changes in function

Alcohol also leads to functional changes in microglia. Cultured primary microglia exposed to ethanol show increased phagocytic activity as measured by ingestion of labeled beads (Fernandez-Lizarbe et al. 2009). Additionally, these cultured microglia showed

increased activation of NF-κB, expression of COX-2 and iNOS, and release of TNF-α, IL-1β and nitric oxide, which are also released in response to LPS. Neurons cultured in the ethanol-treated microglia conditioned media undergo increased apoptosis, suggesting that the molecules secreted by microglia in response to ethanol can cause neuronal cell death (Fernandez-Lizarbe et al. 2009). Although it is hard to attribute *in vivo* changes to only microglia, LPS and ethanol exposure in rodents and mice leads to increased expression of pro-inflammatory molecules known to be released by microglia (TNF-α, IL-1β, iNOS, Il-6, CCL2) as well as increased expression of inflammatory molecules often found in microglia (TLRs, NF-κB, NLRP3 inflammasome) (Lippai et al. 2013; Alfonso-Loeches et al. 2010; Crews et al. 2013; Kane et al. 2013; Whitman et al. 2013). In mice exposed to chronic ethanol, increased expression of COX-2 is co-localized to microglia, although it is seen in neurons and astrocytes as well (Alfonso-Loeches et al. 2010). Increases in cytokines and immune signaling molecules common to microglia are also seen in human post-mortem brains, although the data do not show specific microglial localization (He & Crews 2008; Crews et al. 2013).

1.III.b.iii. Microglial changes in gene expression

Although changes in gene expression have been observed in response to ethanol in cultured microglia (Fernandez-Lizarbe et al. 2009; Fernandez-Lizarbe et al. 2013), none of these experiments have been unbiased gene expression screens and it has recently been acknowledged that cultured microglia have a different gene expression signature than microglia *in vivo* (Butovsky et al. 2013). Additionally, gene expression studies have looked at the transcriptome response to ethanol and identified immune changes (often

attributed to microglia) (Osterndorff-Kahanek et al. 2015; Osterndorff-Kahanek et al. 2013; Most et al. 2015; Lewohl, L. Wang, Miles, Zhang, Dodd & Harris 2000b; Jianwen Liu et al. 2006), but none of these studies have been performed using isolated microglia. Thus, there is a need for further identification of microglia-specific changes in response to ethanol *in vivo* to further characterize the role of microglia in ethanol-induced neuroimmune signaling.

1.III.c. Microglial heterogeneity

Once microglia were determined to be from a macrophage lineage, researchers began to apply knowledge based on macrophages to microglia. However, recent studies have highlighted the differences between macrophages and microglia, as well as the differences between types of microglia used in experiments. Butovsky et al. compared microglia with several immune cells and tissue-specific macrophages and found that all cell types differed, but microglia were most closely related to splenic red-pulp macrophages, followed by other tissue-specific macrophages, then dendritic cells, and furthest from B and T cells (Butovsky et al. 2013). In addition, they discovered that microglial cell lines used *in vitro* (primary microglia, BV2 and N9 microglial cell lines, embryonic stem cell microglia, and RAW264.7 macrophages) do not express the same microglial signature observed in isolated adult microglia. These results highlight the importance of being cautious in our assumptions about microglia moving forward, particularly given the differences in the CNS compared to other tissues (Ransohoff 2016a). This is further evidenced by the recent discovery of important non-immune functions of microglia (1.III.a.i Microglial Function under physiological conditions).

In addition to differences between macrophage sub-types and differences between microglia *in vitro* and *in vivo*, microglia display temporal and spatial heterogeneity. In mice, microglia have different gene expression patterns throughout development, with lower expression of microglial genes during embryonic and postnatal stages and higher expression of microglial genes during late adolescence-young adulthood (P21- 2 months) (Butovsky et al. 2013). Additionally, microglia from aged mice show different morphologies, distribution, function, and gene expression signatures (Orre et al. 2014; Hickman et al. 2013). (Harry 2013). These microglial changes are thought to be unrelated to activation state, but attributed microglial dysregulation that contributes to neurodegeneration (Harry 2013).

It has been known for a while that microglia have different densities and morphologies throughout the brain (Lawson et al. 1990). Given the structural and functional differences in brain regions, it is unlikely that microglia are behaving in the same way across the CNS. Consistent with this notion, a recent gene expression study found that microglia from the cerebellum, cerebral cortex, hippocampus, and striatum exhibited distinct gene expression patterns that change during aging (Grabert et al. 2016). These findings highlight the importance of considering age and brain region when collecting and interpreting microglial data.

Although results from rodent studies are informative in our understanding of microglia, data from human microglia will be important for complete understanding. Technologies are advancing to be able to gather this data and should further elucidate how microglia function during homeostasis and respond to different disease states (Mizee et al. 2017).

1.IV. TOLL-LIKE RECEPTORS

PRRs are the molecules responsible for recognizing PAMPs and DAMPS and initiating an innate immune response. There are several classes of PPRs, including Toll-like receptors (TLRs), which were the first class discovered and are the best characterized (Kawasaki & Kawai 2014). There are 10 identified TLRs in humans, and 12 in mice. TLRs are located either on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10) or on intracellular endosomes (TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, TLR13). Cell surface TLRs recognize a variety of PAMPs, most of which are lipids or proteins found on bacteria (peptidoglycans, lipoproteins). In contrast, endosomal TLRs primarily recognize viral nucleic acids, although some do recognize bacterial nucleic acids as well. Additionally, TLRs recognize endogenous ligands including high-mobility group box 1 (HMGB1), heat shock proteins, extracellular matrix components, and micro RNAs (Yu et al. 2010; Coleman et al. 2017). Due to their importance in immune signaling, TLRs and their signaling components have been a focus of the neuroimmune response to ethanol.

1.IV.a. TLR Signaling pathways

TLRs form homodimers or heterodimers and upon recognition of PAMPs or DAMPs, recruit adapter molecules to the intracellular Toll/IL-1 receptor (TIR) domain (Kawasaki & Kawai 2014). The TIR domain is highly conserved across TLRs and is responsible for downstream signaling. There are five different TIR-domain-containing adaptor molecules that can be recruited to TLRs: Myeloid differentiation primary response protein 88 (MyD88), MyD88-adaptor-like (MAL/TIRAP), TIR-domain containing adaptor protein inducing IFNβ (TRIF/TICAM1), TRIF-related adaptor molecule (TRAM/TICAM2), and sterile α- and armadillo-motif-containing protein (SARM)

(O'Neill & Bowie 2007). Each TLR signals through one or multiple of the adaptor proteins MyD88, MAL, TRIF, and TRAM; while SARM acts as an inhibitor of TRIF signaling.

MyD88 is the primary adaptor molecule and is recruited by all TLRs except TLR3 and signals through the MyD88-dependent pathway (**Fig. 1.3**). In addition to MyD88, MAL is required for TLR2 and TLR4 signaling (O'Neill & Bowie 2007). Once recruited, MyD88 signals through a series of molecules collectively referred to as the MyD88-dependent pathway. Bound MyD88 recruits Interleukin 1 receptor associated kinase 4 (IRAK4) and IRAK1 to form the Myddosome complex (Kawasaki & Kawai 2014). IRAK4 phosphorylates and activates IRAK1 leading to its release from the complex. IRAK1 then recruits and binds TNF receptor associated factor 6 (TRAF6) and the two molecules bind TGF-β-activated kinase 1 (TAK1), TAK1-binding proteins TAB1, and TAB2 (Kawasaki & Kawai 2014). TRAF6, TAK1, and ubiquitin-conjugating enzymes form a complex which undergoes poly-ubiquitination leading to TAK1 activation. TAK1 can then activate two separate pathways, the NF-κB pathway and the MAPK pathway (Kawasaki & Kawai 2014).

Activation of the NF-κB pathway results from TAK1 binding to the inhibitor of nuclear factor Kappa B Kinase (IKK) complex, containing IKKα, IKKβ, and IKKγ (NEMO). The binding of TAK1 to the complex leads to phosphorylation and activation of IKKβ, which in turn phosphorylates inhibitor of NF-κB (IκB) leading to its ubiquitination (Kawasaki & Kawai 2014). Ubiquitination of IκB leads to the release of NF-κB and its translocation to nucleus, where it binds DNA and leads to the transcription of pro-inflammatory cytokines (e.g. IL-1β, TNFα, IL-6, ROS) (Mogensen 2009). TAK1 is also able to activate members of MAPK family, leading to activation of the transcription factor activator protein 1 (AP-1). AP-1 is primarily composed of the Fos and Jun proteins, which

regulate many processes including differentiation, cell-cycle progression, and apoptosis (Hess et al. 2004).

TLR3 and TLR4 signaling can occur via the TRIF-dependent pathway (MyD88-independent pathway) (O'Neill & Bowie 2007). TLR3 signals exclusively through the TRIF-pathway, while TLR4 recruits MyD88-MAL as well as TRIF-TRAM (O'Neill & Bowie 2007). Once recruited, TRIF binds to TRAF6 and TRAF3. TRAF6 then recruits RIP-1, which can activate TAK1 leading to IKK/ NF-κB signaling (Kawasaki & Kawai 2014). Alternatively, TRAF3 can bind to TBK1 and IKKi, leading to phosphorylation and activation of Interferon regulatory factor 3 (IRF3). Activated IRF3 dimerizes and translocates to the nucleus where it binds DNA (Kawasaki & Kawai 2014). IRF3 is a transcription factor that induces expression of type 1 interferon genes (IFN-α, IFN-β). Interferons, in turn, can activate JAK/STAT signaling leading to the transcription of interferon-inducible genes (Takeda & Akira 2004).

Despite signaling through the MyD88-dependent pathway and activating NF-κB, TLR7 and TLR9 are also able to produce type 1 interferons in plasma dendritic cells. This signaling involves IRAK1, IRAK4, TRAF6, TRAF3, and IKKα (Mogensen 2009). In this pathway, IRAK1 phosphorylates and activates IRF7, which can transcribe type 1 interferons.

The TLR signaling pathways are further complicated by negative and positive regulation. The adaptor molecule SARM is produced in response to TRIF-pathway signaling and subsequently acts as a negative regulator of TRIF (O'Neill & Bowie 2007). In the MyD88-dependent pathway, the splice variant MyD88s is non-functional and competes with MyD88 binding, leading to negative regulation of the pathway (O'Neill & Bowie 2007). Additionally, there are several other molecules that inhibit MyD88, TRIF, TRAF6, NF-κB, and IRF3 (Kawasaki & Kawai 2014). Conversely, many cytokine receptor

signaling pathways result in the production of more cytokines, leading to positive feedback and exacerbation of the inflammatory response. One example is IL-1β, a transcriptional product of MyD88-signaling, that also activates the MyD88-dependent pathway itself, leading to positive regulation.

Studies elucidating the TLR signaling pathways have come from the periphery, not the brain. It is assumed that signaling occurs in the same way in the brain, however, that has yet to be proven. There is evidence that TLR signaling can differ in cell types, evidenced by TLR2 induced production of type 1 interferons in plasmacytoid dendritic cells (Kawai & Akira 2010). Future work will be necessary to understand how CNS-specific TLR signaling occurs.

1.IV.b. TLR signaling and ethanol

In addition to increasing immune signaling and microglial activation, ethanol influences TLR signaling, which is unsurprising given the importance of TLRs in the immune response. Within the TLR family, TLR4 has been studied the most in relation to alcohol. Not only have several studies shown that TLR4 expression and signaling is increased in response to ethanol, but there is also substantial evidence that many of the ethanol-induced neuroimmune changes are TLR4 dependent. Recently, studies have investigated the effects of alcohol on other TLRs as well, including TLR2, TLR3, TLR7, and TLR9.

Consuelo Guerri and her group have performed several *in vitro* studies showing how ethanol impacts TLRs. TLR4 and its associated pathways are upregulated in response to ethanol in macrophages, astrocytes, and microglia and this ethanol response involves the clustering of TLR molecules in lipid rafts (Fernandez-Lizarbe et al. 2008; Blanco et al.

2005; Pascual-Lucas et al. 2014; Fernandez-Lizarbe et al. 2009; Alfonso-Loeches et al. 2010). Furthermore, the immune response to ethanol *in vitro* is TLR4 dependent, and is potentiated by TLR2 (Fernandez-Lizarbe et al. 2009; Blanco et al. 2005; Fernandez-Lizarbe et al. 2013). Rodent models of chronic or binge ethanol exposure also show changes in TLRs. Chronic ethanol feeding leads to increased expression and activation of the endogenous ligand HMGB1, increased expression of TLR2, TLR4, TLR9 as well as increased cytokine expression (Lippai et al. 2013; Whitman et al. 2013). Another chronic ethanol study shows increased expression of the co-receptor CD14, activation of NF-κB, and expression of pro-inflammatory mediators that are observed in wild type (WT) mice but not TLR4 knockout mice (Alfonso-Loeches et al. 2010). Furthermore, TLR4 knockout mice don't display the behavior and cognitive defects observed in ethanol-exposed WT mice (M. A. Pascual et al. 2011). Binge ethanol exposure also leads to increased TLR2, TLR3, TLR4, and HMGB1 expression, cytokine expression, and NF-κB activation (Crews et al. 2013; Kane et al. 2013; Crews et al. 2006). It is important to note that TLR changes *in vitro* are dose and time dependent (Fernandez-Lizarbe et al. 2009; Fernandez-Lizarbe et al. 2008; Blanco et al. 2008) while changes *in vivo* are dependent on ethanol exposure (acute vs. chronic), time following exposure (collection when alcohol is on board vs. withdrawal), sex, age, species, and brain region (Alfonso-Loeches et al. 2013; Whitman et al. 2013; Kane et al. 2013; Agrawal et al. 2013). Studies in human post-mortem tissue have reaffirmed the relevance of TLR signaling in alcohol use disorders. Although brain region specific, increases in TLR2, TLR3, TLR4, TLR7 and HMGB1 are seen in post-mortem alcoholic brains (Crews et al. 2013; Coleman et al. 2017).

Functional studies have also been performed to determine if TLR signaling regulates ethanol consumption. Surprisingly, TLR4 knockout mice do not show changes in ethanol consumption, even though the TLR4 ligand LPS increases consumption (Blednov,

Benavidez, et al. 2011; Blednov et al. 2017). However, CD14 (co-receptor for TLR4, TLR2, TLR3, TLR7, TLR9) knockout mice show decreased ethanol consumption in both males and females while TLR2 knockout mice showed decreased consumption in males or females depending on the test (Kawasaki & Kawai 2014; Janot et al. 2008; Blednov et al. 2017). Interestingly, male MyD88 knockout mice showed increased ethanol consumption, possibly due to compensatory activation of the TRIF-dependent pathway.

It is worth noting that knockout mice are effected in all tissues and throughout development, making it hard to differentiate peripheral and CNS effects. Studies using viral injections have shown that knockdown of TLR4 and IKKβ can decrease drinking when delivered in certain brain regions, but not in others

(Harris et al. 2017; Truitt et al. 2016; June et al. 2015; Juan Liu et al. 2011). Future studies with brain-region specific manipulations are necessary to further elucidate how TLR signaling in the CNS regulates ethanol consumption.

1.IV.c. Localization of TLR signaling

TLRs are mainly expressed on antigen-presenting cells, leading to the assumption that they are exclusively in microglia in the CNS (Kawai & Akira 2010). However, this notion has become controversial. There are other glial cell types in the brain that participate in the immune response and studies have shown evidence for TLR expression on astrocytes, oligodendrocytes, and endothelial cells. Recently, there has been evidence of TLR expression on neurons, which further complicates our understanding of how these molecules are working in the brain.

There is widespread support for microglial expression of many TLRs at the mRNA level. In primary human glial cultures expression of *Tlr1-9* was observed in microglia, and

Tlr2 and *Tlr3* expression was also observed in astrocytes and oligodendrocytes (Bsibsi et al. 2002). As previously mentioned, mouse primary microglial and astrocyte cultures express Tlr4, and these cells show altered immune activation when derived from Tlr4 knockout mice (Fernandez-Lizarbe et al. 2009; Fernandez-Lizarbe et al. 2013). Recent gene expression studies on the CNS have further elucidated the localization of Tlrs in the brain. A comparison of isolated mouse CNS cell types shows that *Tlrs* are expressed primarily in microglia, with a few exceptions (Y. Zhang et al. 2014). Tlr3 is primarily expressed in astrocytes, but also expressed in microglia and endothelial cells. This is consistent with several studies that have demonstrated astrocytic expression of Tlr3 (Park et al. 2005; Borysiewicz et al. 2013; Scumpia et al. 2005; Jack et al. 2005). Tlr4 and Tlr12 are expressed mostly in microglia, but also in endothelial cells. Tlr1, Tlr4, Tlr5, Tlr6, Tlr8, and Tlr12 show low expression in astrocytes, while Tlr2, Tlr7, Tlr9, and Tlr13 show no astrocyte expression. Investigation of TLR expression in human microglia and astrocytes also revealed that microglia expressed TLR 1-9, and astrocytes have high levels of TLR3 (Jack et al. 2005). However, this study detected low levels of TLR1, 4, 5, and 9 in astrocytes while TLR2, 6, 7, and 8 were undetectable, suggesting possible differences in expression across species.

The lack of Tlr2 expression in astrocytes is inconsistent with several studies, which is possibly due to species differences or changes that occur during culturing (Kielian 2006). Furthermore, the RNA-sequencing is from the mouse cortex and the cell types are collected at different ages using different methods (Y. Zhang et al. 2014). It is surprising that Tlr4 expression was found to be so low in astrocytes, given the numerous experiments that have studied Tlr4 in astrocyte cultures (Gorina et al. 2010; Blanco et al. 2005; Pascual-Lucas et al. 2014). It is possible that the act of culturing the cells alters the expression profile or that the low expression of Tlr4 in astrocytes is sufficient to cause functional changes (Kielian

2006). Further complicating things, the cell type expression of the downstream signaling molecules differs markedly. For example *Trif* and *Myd88* are mostly expressed in microglia, while *Irf3*, *Irak1*, and *Traf6* are expressed relatively evenly across cell types including neurons (Y. Zhang et al. 2014). This begs the question of how molecules within the same pathway could be expressed in different cell types, and at very different levels. It is possible that these molecules are involved in other signaling pathways in the brain that differ in cell-type specificity.

Despite some discrepancies, localization of *Tlr* mRNA is agreed upon far more than localization of TLR protein. One study looking at mouse primary cultures found that TLR2 was expressed in microglia, astrocytes, and oligodendrocytes but not neurons (Lehnardt 2009). Conversely, other studies have found TLR2, TLR3, and TLR4 protein to be highly neuronal (Crews et al. 2013; S.-C. Tang et al. 2007; Préhaud et al. 2005; Aurelian et al. 2016; Peltier et al. 2010). Additionally, functional studies have provide mixed results about localization of TLR signaling. Several studies provide functional evidence for TLR signaling in microglia (Jack et al. 2005; Olson & S. D. Miller 2004; Fernandez-Lizarbe et al. 2009; Lehnardt et al. 2002). In addition, functional studies support the role of TLR signaling in astrocytes (Borysiewicz et al. 2013; Jack et al. 2005; Park et al. 2005), oligodendrocytes , and endothelial cells (Grace et al. 2014). One study found that NF-κB activity, the output of TLR signaling, is nearly absent from neurons under basal conditions and in response to immune stimuli (Listwak et al. 2013). In contrast, other studies have shown that manipulating TLR signaling in neurons leads to changes in immune response

(Leow-Dyke et al. 2012; June et al. 2015; Truitt et al. 2016; Xing-Jun Liu et al. 2016; P. Mukherjee et al. 2015). Understanding the localization of TLR signaling is a major gap in our understanding of innate immunity in the CNS. In addition, it is difficult to plan future experiments manipulating signaling without a full understanding of cell-type

expression. There is a need for research reconciling these discrepancies in TLR cell-type localization.

1.V. MECHANISMS OF REGULATION

1.V.a. How ethanol regulates immune signaling

Based on recent studies, it is accepted that ethanol can modulate immune signaling, both in the periphery and in the CNS. Although it is not completely clear how ethanol does this, there are some proposed mechanisms, including indirect and direct effects (**Fig. 1.4**).

1.V.a.i. Indirect effects via peripheral immune signaling

Ingested alcohol goes through the digestive system into the intestines where it is converted into acetaldehyde by gut bacteria. In turn, acetaldehyde leads to activation of mast cells, a type of white blood cells that regulate intestinal epithelium physiology and secrete cytokines (Ferrier et al. 2006). Together through multiple mechanisms, alcohol, acetaldehyde and activated mast cells compromise the integrity of the intestinal epithelium, leading to increased intestinal permeability or "leaky gut" (Ferrier et al. 2006). "Leaky gut" causes intestinal bacteria and LPS to enter the blood stream which leads to activation of peripheral blood mononuclear cells (PBMCs) and the secretion of cytokines (Leclercq, De Saeger, et al. 2014). Increased LPS and cytokines in the blood stream leads to an innate immune response in the peripheral organs, particularly in the liver (Leclercq et al. 2012). However, LPS is a large molecule that rarely crosses the blood-brain barrier and no endotoxin is detected in the brain following chronic ethanol exposure (Zou & Crews 2012; Banks & S. M. Robinson 2010). It is instead hypothesized that the peripheral immune activation leads to CNS immune activation, through several different mechanisms.

One mechanism is through the circumventricular organs, which are brain structures with a normal blood brain barrier and include the pineal gland and parts of the pituitary gland. Circumventricular organs can respond to PAMPs in the blood stream and produce pro-inflammatory cytokines, which can then enter the brain by diffusion (Dantzer et al. 2008). In another mechanism, cytokines can activate the vagal nerves, which signal to the brain and cause changes in cytokine production, electrical activity, and parasympathetic activity (Olofsson et al. 2012). A third mechanism involves active transport of cytokines across the blood-brain barrier, initiating a CNS immune response (Mayfield et al. 2013; Dantzer et al. 2008). Specifically, release of TNFα in the periphery triggers an immune response in the brain, leading to activation of NF-κB and AP-1 and increased cytokine production (Qin et al. 2007). A final mechanism of immune signaling from the periphery to the CNS involves perivascular macrophages and endothelial cells that line the blood brain barrier, which express PRRs and secrete inflammatory mediators like prostaglandins (Dantzer et al. 2008; Leclercq et al. 2017).

1.V.a.ii. Direct effects of ethanol on CNS immune signaling

However, there are neuroimmune effects of ethanol that differ from those of LPS, suggesting a periphery-independent mechanism of regulation as well. Because ethanol can get in the brain, it is plausible that ethanol is directly causing an inflammatory response within the CNS. One theory is that ethanol leads to the recruitment of TLRs into lipid rafts, leading to their activation (Fernandez-Lizarbe et al. 2008). Although this has been shown *in vitro*, TLR signaling would still require a ligand to induce this signaling. To this end, work by Fulton Crews and colleagues demonstrated that alcohol increases expression of high mobility group box 1 (HMGB1) within the brain, while LPS does not (Whitman et al.

2013). HMGB1, an endogenous ligand for several TLRs, is released by damaged neurons and leads to the induction of pro-inflammatory cytokines (Faraco et al. 2007). Furthermore, HMGB1 antagonism reduced ethanol-induced cytokine induction and neurotoxicity in brain slice cultures (Whitman et al. 2013; Coleman et al. 2017). Recent data also suggest a role for miRNAs in mediating the effects of ethanol in immune signaling. For example, ethanol increased the binding of the miRNA let-7b to HMGB1 and increased their release from microglial microvesicles (Coleman et al. 2017). Ethanol-induced release of let-7b (a TLR7 ligand) increases TLR7 expression, NF-κB activation, and neurodegeneration (Coleman et al. 2017). Together, these data are increasing our understanding of how ethanol activates immune signaling in the brain.

1.V.b. How immune signaling regulates consumption

Ethanol-induced immune signaling is hypothesized to increase consumption leading to more inflammation in a feed forward loop (**Fig. 1.5**). This hypothesis is supported by evidence that alcohol increases immune signaling *(1.II.b. Alcohol's impact on immune signaling*), and that immune signaling alters consumption (*1.II.d. Immune signaling regulates ethanol consumption)*. Immune signaling in the brain, particularly the PFC, is believed to regulate ethanol consumption by altering cortical function. Although not completely understood, there are several proposed mechanisms by which signaling alters cortical function and therefore regulates drinking. These mechanisms include changes in neurotransmitter release and synaptic activity, altered synaptic plasticity, perturbed neuroendocrine function, decreased neurogenesis, and neuronal damage and death.

1.V.b.i. Changes in neurotransmitter release and synaptic activity

Increased neuroimmune signaling leads to the secretion of pro-inflammatory mediators, including cytokines and chemokines. Cytokines and chemokines can modulate the release of several neurotransmitters that contribute to addiction, including glutamate, GABA, dopamine, and serotonin (Cui et al. 2014). In addition, it is well established that pro-inflammatory signaling can alter mood and cause depression (Dantzer et al. 2008). Given the co-morbidity between depression and AUDs and the contribution of negative affect to addiction, mood changes are thought to be an important mediator of immune-induced alcohol consumption (Koob & Volkow 2010; Crews et al. 2011).

A leading hypothesis about how immune signaling regulates alcohol consumption involves increases in cortical hyperexcitability (Crews et al. 2011). The neurotransmitter glutamate regulates excitatory synaptic transmission between the cortex and limbic brain regions (Vetreno & Crews 2014). Studies suggest that chronic alcohol leads to a hyper-glutamatergic state that results in loss of cortical flexibility and increased addiction-like behavior (Gruber et al. 2010), and that immune signaling could mediate this response (Crews 2012; Vetreno & Crews 2014). The cytokine TNFα is released in response to immune activation and reduces the activity of glutamate transporters in astrocytes, while activation of chemokine receptors increases astrocyte glutamate release (Zou & Crews 2005b; Cui et al. 2014). Astrocytes are responsible for glutamate uptake from the extracellular space, thus, reduced glutamate transporter activity and increased glutamate release leads to increased extracellular glutamate (Murphy-Royal et al. 2017). In addition, microglia can produce quinolinic acid, which also promotes glutamate release (Kovács 2017). Increases in glutamate accumulation in the synapse cause increased neuronal excitation, which increases cortical excitability and interferes with the ability to differentiate real signal from noise (Crews et al. 2006).

Activity of GABA, the primary inhibitory neurotransmitter, is increased during acute ethanol exposure but decreased with chronic ethanol (Most, Ferguson, et al. 2014). Although ethanol can directly bind the GABA receptor and modulate activity, studies suggest that immune signaling can also influence GABAergic activity. In mouse central amygdala, ethanol increased presynaptic and postsynaptic GABA transmission, and this was diminished in CD14 knockout mice, suggesting immune regulation (Bajo et al. 2014). The amygdala signals to the cortex and is important for the motivation effects of alcohol use, thus altered amygdalar GABAergic activity could influence decision making and drinking behavior. Furthermore, chemokines (CCL2, CXCL-12) and cytokines (TNFα, IL-1β) regulate release of GABA and trafficking of GABA receptors (Cui et al. 2014; Galic et al. 2012). IL-1β, for example, has been shown to decrease or increase GABAergic activity depending on the conditions (Galic et al. 2012). In turn, GABA can regulate the release of inflammatory cytokines, so decreased GABA activity can further exacerbate inflammation (A. H. Miller et al. 2013).

Dopamine is neurotransmitter that mediates reward and is highly implicated in addiction. There is evidence that neuroimmune signaling regulates dopamine synthesis, re-uptake, and release. MAPK signaling, which is activated in response to immune signaling, increases dopamine reuptake, reducing levels of extracellular dopamine (A. H. Miller et al. 2013). In addition, immune activation decreases release of the dopamine precursor dopa (A. H. Miller et al. 2013).

Serotonin is a monoamine neurotransmitter that is implicated in anxiety, depression, and addition. Inflammatory cytokines activate the enzyme IDO (Indoleamine-2,3-dioxygenase) that converts tryptophan (the primary amino acid component of serotonin) into kynurenine, reducing serotonin levels (A. H. Miller et al. 2013). Cytokines can also impair activity of BH4, a co-factor involved in the synthesis of serotonin and

dopamine. In addition to altering serotonin synthesis, MAPK signaling and cytokines can also increase activity of the serotonin transporter (SERT), decreasing the level of extracellular serotonin.

1.V.b.ii. Changes in synaptic plasticity: LTD and LTP

Another hypothesis about how immune signaling promotes addition is that neuroimmune signaling causes changes in LTP and LTD, leading to altered learning and memory. Because microglia are now known to influence LTP and LTD during homeostasis, their shift to an inflammatory state could lead to neglect of their homeostatic roles and impaired learning and memory (Vetreno & Crews 2014). Supporting this hypothesis, peripheral injections of LPS produce transient deficits in LTP, while long-term LPS exposure causes persistent deficits in LTP (Maggio et al. 2013). Specific immune molecules have been shown to influence LTP and LTD, including IL-1β, MHCI, TNFα (Shatz 2009; Cui et al. 2014). Therefore, the neuroimmune response could be have a role in the changes in synaptic plasticity seen following chronic alcohol use (McCool 2011). Impaired learning and memory, in turn, can further promote consumption.

1.V.b.iii. Altered neuroendocrine function

In addition to modulating neurotransmitter activity, increased immune signaling also perturbs neuroendocrine signaling via the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is often dysregulated with AUDs and stress is a major trigger for relapse (Koob & Volkow 2010). Cytokines and chemokines can regulate the release of stress hormones like cortisol, while stress-induced glucocorticoids can in turn regulate cytokine expression (Cui et al. 2014). Interestingly, many of the immune changes seen in response

to ethanol are also seen in response to stress (Crews 2012; Knapp et al. 2016). Studies have demonstrated that injection of immune activators into the brain increases ethanol withdrawal-induced anxiety-like behavior (Breese et al. 2008). Furthermore, ethanol-withdrawal induced cytokine expression is blocked by a CRF1 receptor antagonist (Whitman et al. 2013). Therefore, perturbation of immune signaling by ethanol could alter the stress and anxiety response and drive further use.

1.V.b.iv. Decreases in neurogenesis

There is evidence that innate immune signaling leads to inhibition of neurogenesis (*also covered in 1.II.c. Immune signaling and ethanol-induced damage*), which is linked to drug induced negative-affect and depression-like behavior (Crews et al. 2011). Supporting the idea that TLR activation reduces neurogenesis, activation of TLR2 or TLR3 signaling in embryonic cortical neural stem cells inhibits progenitor cell proliferation (Lathia et al. 2008; Okun et al. 2010). There is evidence for this action in the adult mouse as well, where TLR4 activation also inhibits progenitor cell proliferation in the hippocampus (Rolls et al. 2007). Furthermore, increased extracellular glutamate leads to NMDAR activation, decreasing brain derived neurotrophic factor (BDNF), a regulator of neurogenesis (A. H. Miller et al. 2013). Interestingly, some cytokines promote neurogenesis (IL-1β, CXCL-12), while others inhibit it (TNFα, IL-6) (Cui et al. 2014). Reduced neurogenesis is associated with impaired learning, memory, and mood regulation (Vetreno & Crews 2014; Kovács 2017). Furthermore, reduced neurogenesis means a reduction in neuronal turnover which could contribute to more pervasive drug-associated memories (Kovács 2017). Recovery from AUDs is accompanied by increased

neurogenesis and improved mood and cognition, suggesting that altered neurogenesis could contribute to cognitive deficits seen in alcoholics (Crews et al. 2011).

1.V.b.v. Neuronal damage and death

As previously covered in 1.II.c., ethanol-induced immune signaling can cause neuronal damage and neurodegeneration (Alfonso-Loeches et al. 2010). It is hypothesized that this damage contributes to cortical dysfunction and addictive behavior. Increased cytokine production in astrocytes results in a hyper-glutamatergic state *(see 1.V.b.i. Changes in neurotransmitter release and synaptic activity*). Not only does hyperexcitability increase neuronal excitation, but it also causes excitotoxicity: neuronal damage or death due to over activation of glutamate receptors (Crews et al. 2011).

Another mechanism for neuronal death involves reactive oxygen species (ROS), which are a byproduct of alcohol metabolism and a product of immune signaling. ROS are secreted by activated microglia and lead to oxidative stress and neuronal death (Crews et al. 2015). NADPH oxidase, the enzyme that is responsible for the formation of reactive oxygen species, is also induced by ethanol and LPS (Crews et al. 2015). Increased NADPH activity leads to oxidative stress, neurodegeneration, and increased NF-κB transcription (Qin et al. 2013). Alcohol exposure also decreases antioxidant levels and activity, reducing the body's ability to combat oxidative stress (Crews et al. 2015). Dopaminergic neurons are particularly sensitive to increases in oxidative stress (O'Callaghan et al. 2008) and play and important role in addictive behaviors, suggesting that damage in these neurons could influence consumption (Qin et al. 2007).

Additional work suggests that the cytokine TGF-β could also be a mediator of neuronal apoptosis. TGF-β1 plays an important role in alcohol-induced liver injury

(Dooley & Dijke 2012) and levels are increased in the blood of alcohol dependent subjects (Kim et al. 2009). Ethanol-induced TGF-β1 has been shown to cause neuronal cell death by elevating pro-apoptotic proteins, lowering levels of anti-apoptotic proteins, and increasing production of caspase 3 (Crews et al. 2015). Although implicated as an anti-inflammatory cytokine, studies show that NF-κB activity mediates TGF-β stimulation, highlighting the its potential role in pro-inflammatory cell responses (Tobar et al. 2010).

Additionally, ethanol-induced immune signaling leads to increased NF-κB binding and decreased CREB-DNA binding. CREB targets many genes that are responsible for responsible for promoting neuronal survival and preventing excitotoxicity and apoptosis (Vetreno & Crews 2014). While alcohol decreases CREB activity, activated glial cells cause activation of NF-κB and increases in extracellular glutamate. The shift in the CREB/ NF-κB balance results in hyperexcitability, cytokine induced-neuronal damage and cell death, and altered neuronal function, which can all contribute to addiction (Vetreno & Crews 2014; Kovács 2017).

1.V.b.vi. Consequences of altered cortical function

In summary, there are several potential mechanisms by which ethanol-induced immune signaling regulates brain activity and thus addictive behavior. It is likely that these processes are occurring in parallel and that modulating immune signaling upstream could improve many functions at once. Although immune signaling is altered in many brain regions, the sensitivity of the PFC to these changes is thought to be an important mechanism by which immune signaling facilitates addictive behavior. The importance of the PFC in addiction is discussed in detail in *section 1.I.a. The Prefrontal Cortex and AUDs*. In addition to mediating critical behaviors like impulse inhibition, decision making,

and reversal learning, the PFC integrates information from other brain regions that regulate reward (nucleus accumbens, ventral tegmental area), negative affect (amygdala), and learning and memory (hippocampus).

1.VI. Goals of Dissertation

The main goal of my dissertation was to elucidate the neuroimmune response to chronic voluntary ethanol in a mouse model. In short, I found that voluntary ethanol has several important effects on neuroimmune signaling in the PFC, many of which are novel or unexpected.

First, I found that chronic ethanol induces expression and signaling of the TRIF-dependent pathway *in vivo* in a time- and brain-region specific manner. TRIF-dependent immune changes were increased in the PFC and nucleus accumbens, while they were decreased in the amygdala. I then demonstrated that inhibition of TRIF-dependent signaling using an IKKε/TBK1 inhibitor can decrease ethanol consumption.

Then, to determine which cell types are responsible for neuroimmune changes, I profiled the cell-type localization of components and outputs of the TLR signaling pathways. I discovered that most the neuroimmune mRNAs are localized in microglia and increase in microglia in response to LPS. I also discovered that many of the microglial gene expression changes were missed using a typical preparation. Although unable to reliably determine the protein localization for the same molecules, I did reveal some potential reasons why this is disagreed upon

Based on my revelation that most immune genes were microglial, but missed in a typical preparation, I aimed to profile the microglial response to ethanol. To determine the microglia-specific response to ethanol, I used isolated microglia and compared the

transcriptome changes to those observed in the total homogenate. I found that microglia have distinct gene co-expression networks and that many of the changes detected in microglia are not observed in the total homogenate. Specifically, microglia showed many genes related to endosomal TLR signaling and TGF-β signaling, validating our interest in TRIF-dependent signaling and suggesting a role for growth factor signaling in ethanol-induced neuroimmune signaling.

I then attempted to profile the microglial response 1-week after peripheral LPS treatment, a time point that previously showed high gene expression overlap with ethanol consumption in the total homogenate. I found that LPS not only increased expected cytokine genes in microglia, but also increased several many ribosomal genes and genes related to misfolded proteins. I further compared the microglial response to LPS and ethanol and found that while both treatments modulated immune signaling, the genomic responses were very different.

Together, the data I present provide new targets to investigate in the ethanol-neuroimmune response. Future studies in the lab will build on these experiments.

1.VII. FIGURES

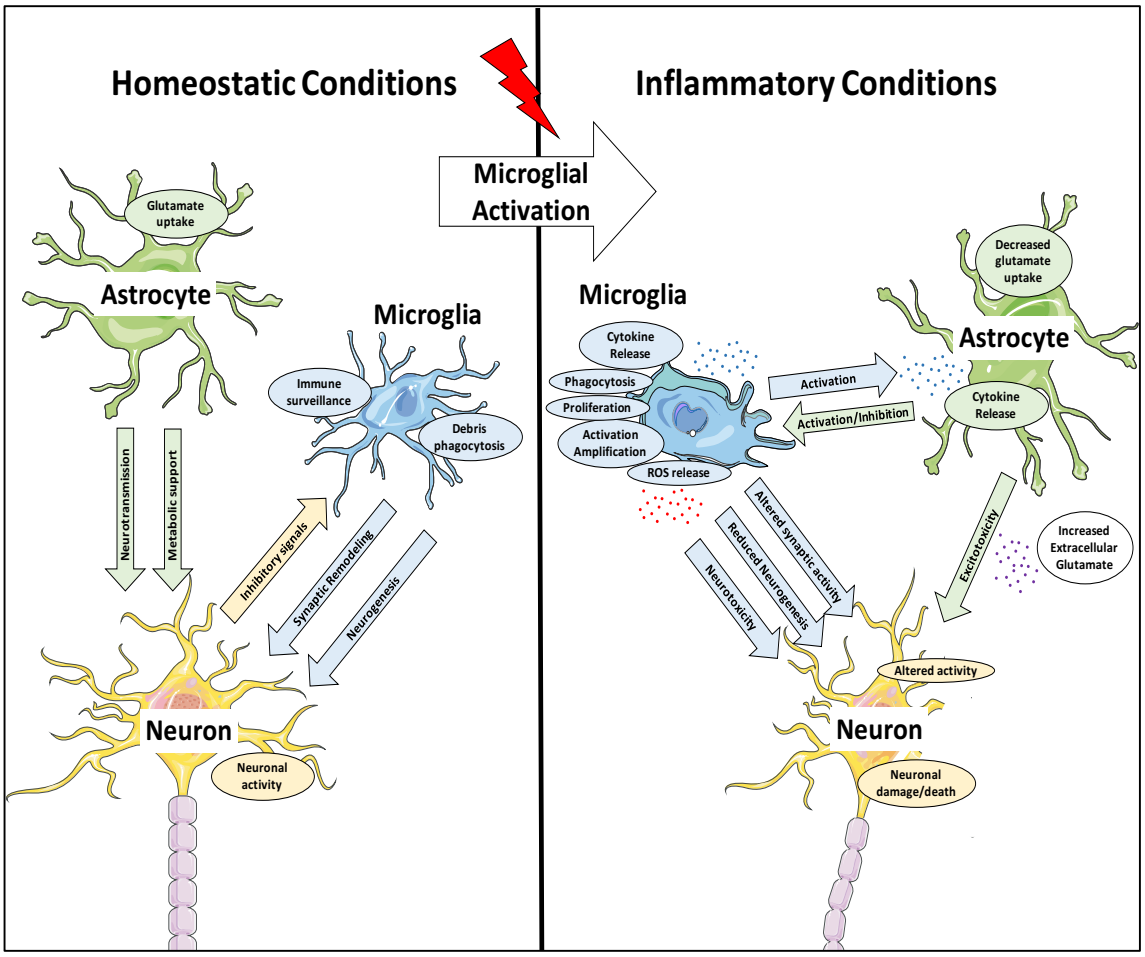


Figure 1.1: Microglial function in homeostasis and inflammation

Under homeostatic conditions, microglia are ramified with small cell bodies and long processes that survey the brain for damage signals. In addition, microglia regulate neurogenesis and interact with neurons influencing synaptic activity. In turn, neurons send inhibitory signals to microglia preventing activation. In response to activation, microglia change morphology and have a larger cell body and smaller processes. Although microglial activation is heterogeneous, some effects include release of cytokines and reactive oxygen species (ROS), proliferation, and phagocytosis. Cytokines, in turn, can activate astrocytes, which further modulate the microglial response. In addition, astrocyte activation leads to decreased glutamate uptake and increased extracellular glutamate, which can alter neuronal activity and cause excitotoxicity. Cytokines and ROS can cause neurotoxicity, while activated microglia neglect their functions in neurogenesis and synaptic plasticity.
Figure created using http://servier.com/Powerpoint-image-bank

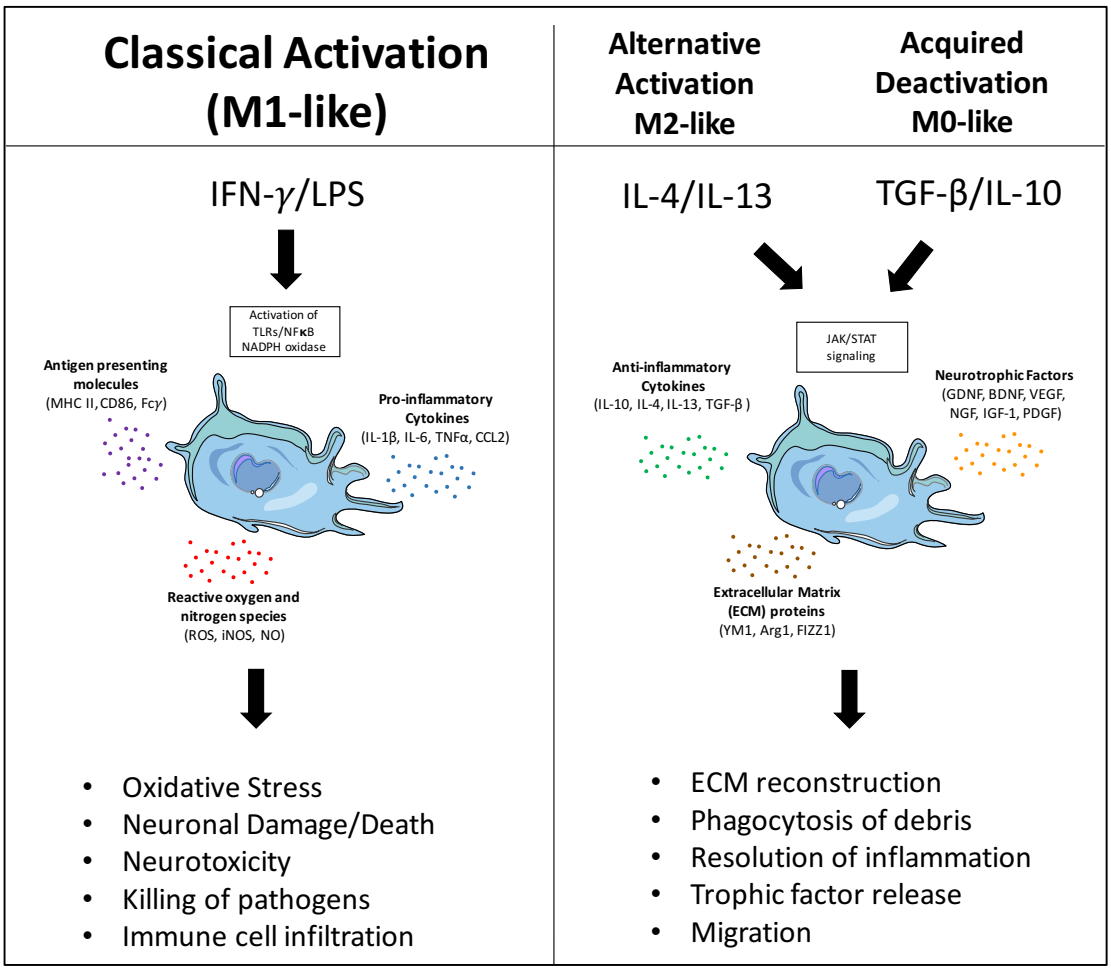


Figure 1.2: Microglial activation states

Although controversial, it is believed that microglia have a classical pro-inflammatory (M1-like) and alternative anti-inflammatory (M0/M2-like) response. Classical activation occurs in response to IFN-γ/LPS *in vitro* and leads to activation of TLRs and NF-κB. A classical M1-like response leads to increases in antigen presenting molecules and secretion of pro-inflammatory cytokines and reactive oxygen species. The function of an M1-like response is to kill pathogens, but it can also lead to neurotoxicity. *In vitro*, alternative activation (M2-like) occurs in response to IL-4/IL-13 while acquired deactivation occurs in response to TGF-β/IL-10. Both sets of inducers lead to the production of anti-inflammatory cytokines, neurotrophic factors, and extracellular matrix proteins. This response is usually reparative and includes phagocytosis of debris and resolution of inflammation.
Figure created using http://servier.com/Powerpoint-image-bank

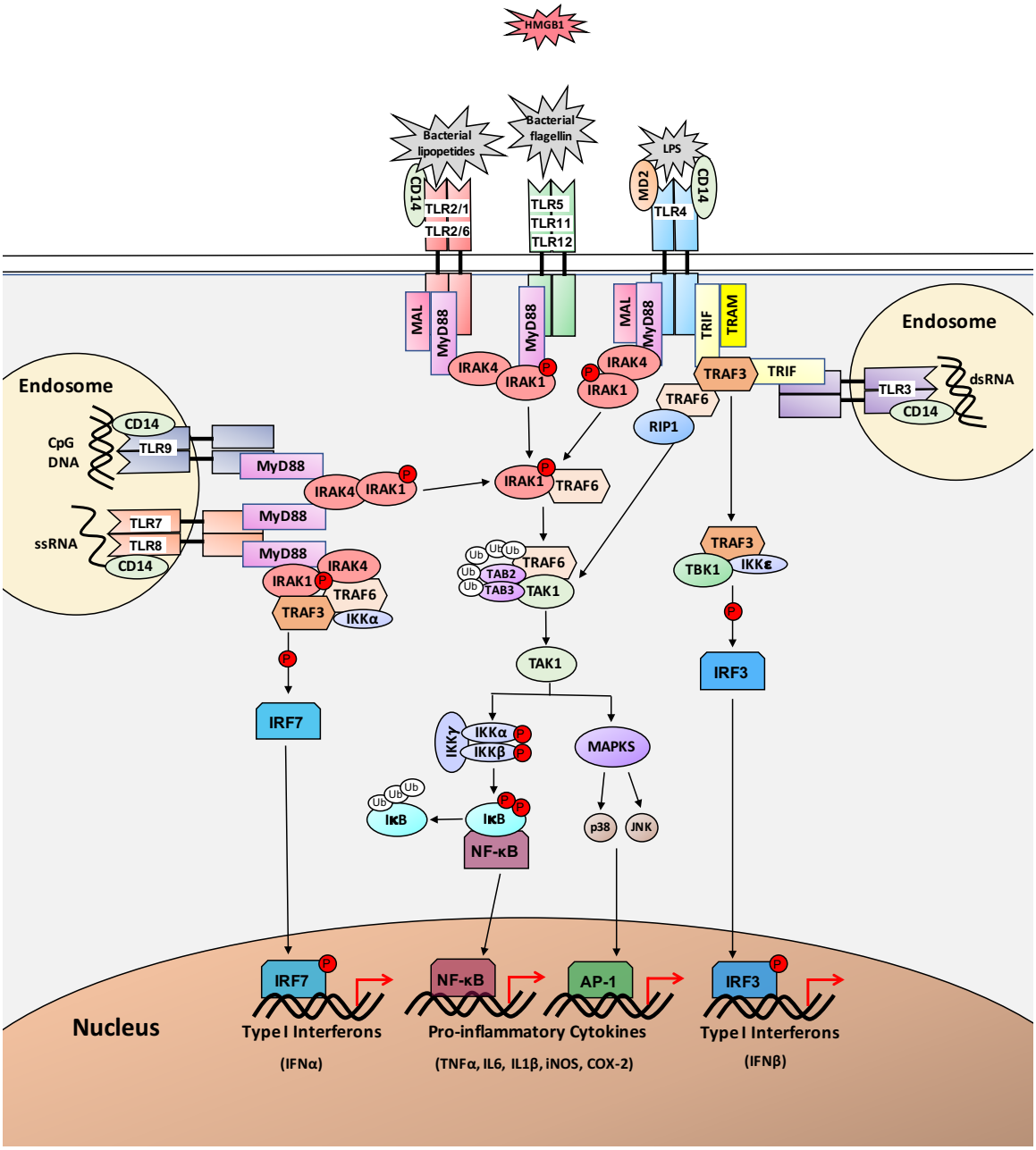


Figure 1.3: TLR signaling pathways

Figure 1.3: continued

Schematic of TLR signaling in response to their respective ligands or DAMPS (such as HMGB1 or heat shot proteins). All TLRs except TLR3 signal through the canonical MyD88-dependent pathway. In this pathway, MyD88 recruits IRAK4 and IRAK1, leading to the phosphorylation and activation of IRAK1. Phosphorylated IRAK1 then dissociates and binds TRAF6 and ubiquitin conjugating enzymes, leading to polyubiquitination of TRAF6. The polyubiquitin chains then bind TAB2 and TAB3, leading to activation of TAK1. Activated TAK1 signals through either IKKs or MAPKs. TAK1 can phosphorylate IKKs which in turn phosphorylate IκB leading to its ubiquitination and the release of NF-κB. NF-κB then translocates to the nucleus where it transcribes proinflammatory genes. Alternatively, TAK1 can induce MAPK kinases and activate p38 and JNK leading to activation of the transcription factor AP-2. Instead of the MyD88-dependent pathway, TLR3 signals through the TRIF-dependent pathway, while TLR4 signals through both pathways. The TRIF-dependent pathway utilizes the adaptor molecule TRIF which binds to TRAF3 and TRAF6. TRAF6 can then activate TAK1 activation through RIP1. In contrast, TRAF3 binds TBK1 and IKKε leading to phosphorylation and activation of IRF3. IRF3 then translocates to the nucleus where it leads to transcription of type I interferons and interferon inducible genes. TLR7/8/9 can also drive transcription of interferon inducible genes by signaling through the MyD88-dependent pathway. This signaling involves the recruitment of IRAK1, IRAK4, TRAF6, TRAF3 and IKKα. This complex leads to the phosphorylation and activation of IRF7 which translocates to the nucleus and transcribes type I interferons.

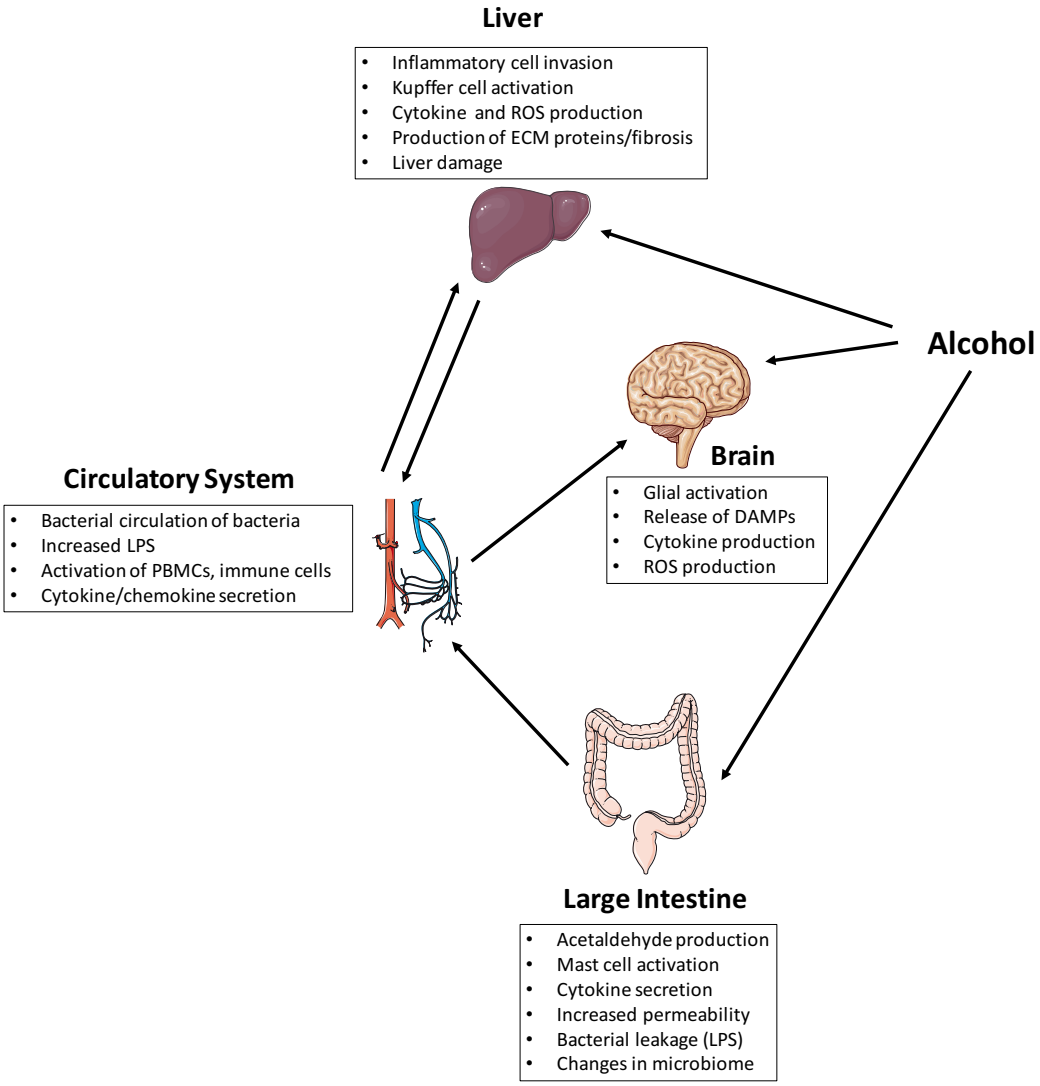


Figure 1.4: Alcohol effects on gut-liver-brain immune signaling

Alcohol can affect immune signaling in the brain both directly and indirectly. Ingested alcohol reaches the intestines where it can cause increased intestinal permeability, bacterial leakage, and cytokine secretion. Bacteria and cytokines then enter the blood stream, where the inflammatory response is amplified by peripheral blood mononuclear cell (PBMC) activation and cytokine secretion. Inflammatory cells and cytokines then reach the liver where Kupffer cells become activated and liver damage can occur over time. Increased cytokine secretion from the liver re-enters the blood stream and cytokines can enter the brain by crossing the blood brain barrier. In addition, alcohol can directly enter the brain and cause the release of endogenous TLR ligands and promote TLR binding in lipid rafts. Figure created using http://servier.com/Powerpoint-image-bank

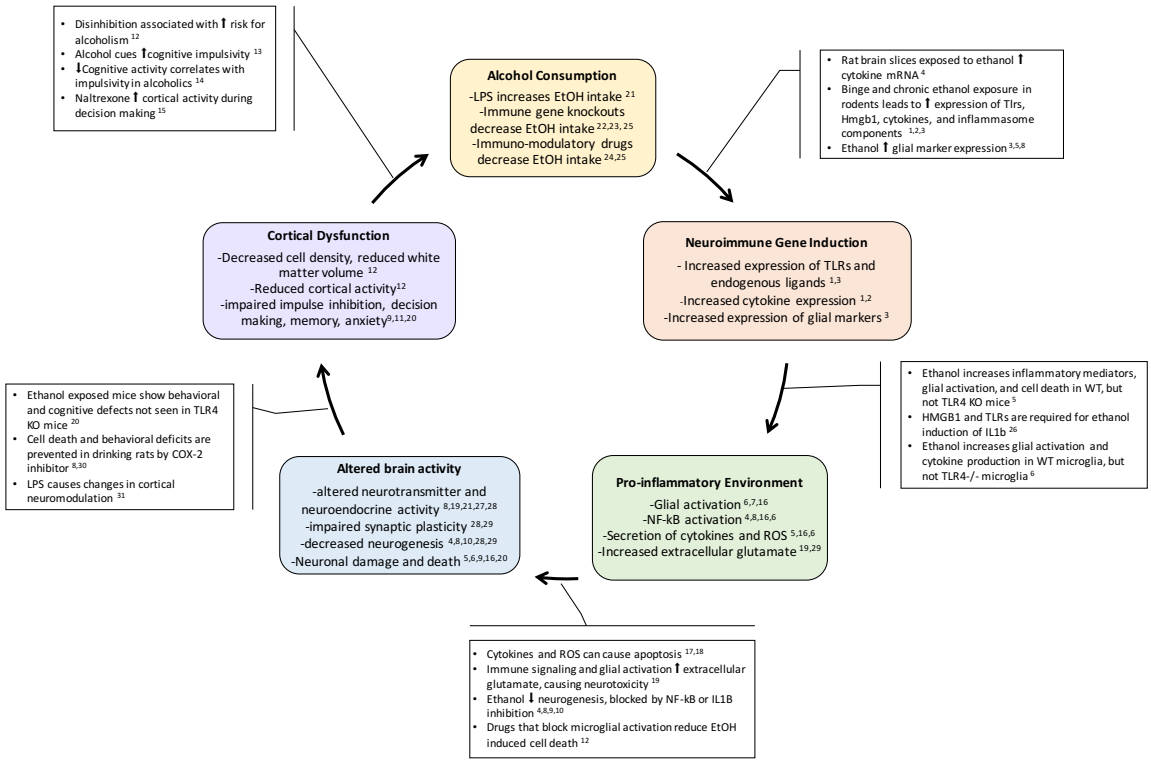


Figure 1.5: Hypothesized alcohol-immune feed forward loop

Figure 1.5: continued

It is hypothesized that chronic alcohol consumption leads to neuroimmune gene induction, resulting in a pro-inflammatory environment, altered brain activity and ultimately cortical dysfunction, which leads to increased consumption. This figure displays this hypothesized loop with specific examples and references for each stage, as well as evidence that each stage causes the next one (boxes by arrows).

References: **1:** (Whitman et al. 2013), **2:** (Kane et al. 2013), **3:** (Lippai et al. 2013), **4:** (Zou & Crews 2010), **5:** (Alfonso-Loeches et al. 2010), **6:** (Fernandez-Lizarbe et al. 2009), **7:** (He & Crews 2008), **8:** (Crews et al. 2006), **9:** (Crews & Boettiger 2009b), **10:** (Kimberly Nixon & Crews 2002), **11:** (Abernathy et al. 2010), **12:** (Oscar-Berman & Marinković 2007), **13:** (Noël et al. 2007), **14:** (Boettiger et al. 2007), **15:** (Boettiger et al. 2009), **16:** (Blanco et al. 2008), **17:** (Hsu et al. 1996), **18:** (Kamata et al. 2005), **19:** (Zou & Crews 2005b), **20:** (M. A. Pascual et al. 2011) **21:** (Blednov, Benavidez, et al. 2011), **22:** (BLEDNOV et al. 2005), **23:** (Blednov, Ponomarev, et al. 2011), **24:** (Agrawal, Hewetson, C. M. George, Syapin & Bergeson 2011b), **25:** (Truitt et al. 2016), **26:** (Crews et al. 2013) **27:** (Dantzer et al. 2008) **28:** (Cui et al. 2014) **29:** (Vetreno & Crews 2014) **30:** (M. Pascual et al. 2007) **31:** (Ming et al. 2015)

Chapter 2: Chronic Ethanol Consumption: Role of TLR3/TRIF-dependent signaling

Gizelle M. McCarthy B.S., Anna S. Warden B.S., Courtney R. Bridges B.S., Yuri A. Blednov, PhD, R. Adron Harris, PhD

2.I. Abstract

Chronic ethanol consumption stimulates neuroimmune signaling in the brain, and toll-like receptor (TLR) activation plays a key role in ethanol-induced inflammation. However, it is unknown which of the TLR signaling pathways, the MyD88 (myeloid differentiation primary response gene 88) dependent or the TRIF (TIR-domain-containing adapter-inducing interferon-β) dependent, is activated in response to chronic ethanol. We used voluntary (every-other-day) chronic ethanol consumption in adult C57BL/6J mice and measured expression of TLRs and their signaling molecules immediately following consumption and 24 hours after removing alcohol. We focused on the prefrontal cortex (PFC) where neuroimmune changes are the most robust, but also investigated the nucleus accumbens (NAc) and amygdala (AMY). *Tlr* mRNA and components of the TRIF-dependent pathway (mRNA and protein) were increased in the PFC 24 hours after ethanol and *Cxcl10* expression increased 0 hours after ethanol. Expression of *Tlr3* and TRIF-related components increased in the NAc, but slightly decreased in the AMY. In addition, we demonstrate that the IKKε/TBK1 inhibitor

Amlexanox decreases immune activation of TRIF-dependent pathway in brain and reduces ethanol consumption, suggesting the TRIF-dependent pathway regulates drinking. Our results support the importance of TLR3 and the TRIF-dependent pathway in ethanol-induced neuroimmune signaling and suggest that this pathway could be a target in the treatment of alcohol use disorders.

2.II. INTRODUCTION

Chronic alcohol consumption leads to adaptive changes in the central nervous system (CNS) that contribute to dependence and the development of alcohol use disorders (AUDs) (Harper 2009; Diamond & Gordon 1997). The prefrontal cortex (PFC), a brain region central to cognitive behavior, is important in the development of AUDs (S. Mukherjee et al. 2008; Abernathy et al. 2010). Chronic alcohol use produces molecular adaptations in the innate immune system and the PFC is particularly sensitive to these changes (NIAAA 2012; Goral et al. 2008; Stolyarova et al. 2015). Although the PFC shows the strongest immune response to ethanol, the nucleus accumbens (NAc) and amygdala (AMY) are also affected (He & Crews 2008; Osterndorff-Kahanek et al. 2015; Bajo et al. 2014). There is evidence that neuroimmune mechanisms cause CNS damage and also promote alcohol dependence, suggesting that these pathways are potential therapeutic targets for the deleterious effects of alcohol (Blednov, Benavidez, et al. 2011; Zou & Crews 2010; Alfonso-Loeches et al. 2010; Blednov, Ponomarev, et al. 2011).

Toll-like receptors (TLRs) are innate immune proteins and key components of the neuroimmune response to ethanol, particularly TLR2, TLR3, and TLR4 (Alfonso-

Loeches et al. 2010; Crews et al. 2013). TLRs are pattern recognition receptors and their signaling is triggered through a variety of pathogen-derived ligands. TLR2 and TLR4 are found on the cell surface and respond to bacterial ligands while TLR3 and TLR7 are in endosomes and respond to viral ligands (Lehnardt 2009). In addition, TLRs can respond to endogenous ligands like high mobility group box 1 (HMGB1) (Anggayasti et al. 2017; Crews et al. 2013). TLRs signal through two pathways, the myeloid differentiation primary response (MyD88) and the TIR-domain-containing adapter protein (TRIF/TICAM-1) pathway. In the MyD88-dependent pathway, the adapter protein MYD88 binds to the Toll/IL-1 receptor (TIR) domain and recruits interleukin-1 (IL-1) receptor associated kinase 4 (IRAK4), IRAK1, and TNF receptor-associated factor 6 (TRAF6). IRAK4 then phosphorylates IRAK1, which leads to the release of TRAF6 and the formation of the TRAF6 complex. Members of the TRAF6 complex phosphorylate the inhibitor of nuclear factor kappa-B kinase (IKK) complex, allowing release and activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Takeda & Akira 2005). NF-κB activation leads to the transcription of proinflammatory cytokines, such as IL-1β, tumor necrosis factor alpha (TNF-α), and interleukin 6 (IL-6). The TRIF-dependent pathway uses the adapter protein TRIF, which signals through IKKε (also referred to as IKKi) and TANK-binding kinase 1 (TBK1), leading to the phosphorylation of interferon regulator factor 3 (IRF3) and the transcription of type I interferons. Activation of the TRIF-dependent pathway also leads to increased transcription of interferon inducible genes like C-X-C motif chemokine 10 (Cxcl10/IP-10) and Chemokine (C-C motif) ligand 5 (Ccl5/RANTES) (Hirotani et al. 2005;

Weighardt et al. 2004). All TLRs (except TLR3) signal through the MyD88-dependent pathway while TLR3, TLR4, and possibly TLR2 signal through the TRIF-dependent pathway (Nilsen et al. 2015; Takeda & Akira 2005).

Although studies have shown increased TLR mRNA and protein levels with high dose ethanol exposure, few have examined the effects of voluntary ethanol consumption on the MyD88 vs. TRIF pathway components in the brain (Whitman et al. 2013; Lippai et al. 2013; Crews et al. 2013). It is important to understand the downstream signaling events that mediate ethanol-induced changes in order to target the relevant pathways and simultaneously alter the activity of multiple TLRs. The goal of this study was to examine TLR signaling components after voluntary chronic ethanol consumption.

Because TLRs are involved in many immunological conditions, there is an effort to find drugs that modulate their signaling. Most drugs targeting the TRIF-dependent pathway work by inhibiting IKKε, TBK1, or both (Hasan & Yan 2016). Amlexanox, an FDA-approved treatment for the treatment of aphthous ulcers in the US and is used to treat a variety of inflammatory diseases in Japan. Amlexanox is a dual TBK1 and IKKε inhibitor that has reduced inflammation and hepatic steatosis in obese mice, suggesting that it could have therapeutic uses for other inflammatory conditions that promote TRIF-pathway signaling (Reilly et al. 2013).

We investigated both mRNA and protein changes in TLRs and components of the MyD88- and TRIF-dependent pathways using C57BL/6J mice undergoing an every-other-day 2-bottle choice (EOD-2BC) paradigm, which is a voluntary drinking test that leads to escalation in drinking and changes in gene expression (Osterndorff-Kahanek et al. 2013;

Crabbe et al. 2012). We examined three different brain regions and two different time points to compare changes that occur immediately after consumption, while alcohol is still present in low levels, with those that occur when the mice are anticipating reinstatement of alcohol. We present novel evidence for TRIF- and brain region-dependent signaling during alcohol withdrawal that may influence craving. To determine the functional importance of TRIF-signaling in regulating ethanol consumption, we show that Amlexanox inhibits the inflammatory response in brain and reduces ethanol consumption. Our results suggest that TRIF-dependent may have a role in the development of AUDs and is a potential therapeutic target.

2.III. Materials and Methods

2.III.a. Ethics Statement

All procedures were approved by the University of Texas at Austin Institutional Animal Care and Use Committee (animal protocol number AUP-2013-00061) and adhered to the NIH Guidelines. The University of Texas at Austin animal facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

2.III.b. Animals and voluntary ethanol consumption

Studies were conducted in adult (6-8 weeks old) drug-naïve C57BL/J (B6) male mice (Jackson Laboratories, Bar Harbor, ME). Mice were individually housed and allowed to acclimate to upright bottles one week before the start of the experiment. The

experimental rooms were maintained at an ambient temperature of 21±1°C, 40-60% humidity, and a regular light/dark schedule (7 AM-7 PM). Food and water were available *ad libitum*.

An EOD-2BC paradigm was used (Osterndorff-Kahanek et al. 2013; Crabbe et al. 2012), and mice were randomly assigned to the control or treatment group. Treatment and control groups each contained 15 mice per time point (60 total). Control groups had access to water every day. Treatment groups had EOD access to water and a 15% (v/v) ethanol solution, and water only on off days. On days that ethanol was available, bottle positions were alternated to control for potential side preferences. Ethanol and water bottles were weighed after drinking days and animals were weighed once per week to calculate consumption (**Fig. 2.6**). The study concluded after 60 days (30 drinking days).

2.III.c. Blood Alcohol Measurements and Tissue Harvest

Mice were sacrificed either immediately after ethanol was removed (0-hour group) or 24 hours after ethanol was removed (24-hour group). For the 0-hour group, retro-orbital bleeds were performed before sacrifice to determine blood alcohol concentration (BAC). BAC values, expressed as mg ethanol per deciliter of blood (**Table 2.1**), were determined spectrophotometrically by an enzyme assay (LUNDQUIST et al. 1959).

Five mice per group were used for immunohistochemistry. These mice were anaesthetized using isofluorane and given intraperitoneal injections of Euthanasia III Solution (TW Medical, Lago Vista, TX). Once death was confirmed, transcardial

perfusion was begun with PBS and followed by 4% paraformaldehyde (PFA). The whole brain was removed and post-fixed in 4% PFA. The remaining 10 mice per group were used for RNA and protein isolation. Following rapid cervical dislocation, brains were removed and placed on ice. The PFC was dissected as previously described (Osterndorff-Kahanek et al. 2013), cut in half, and flash frozen in liquid nitrogen. The remainder of the brain was flash frozen separately.

2.III.d. Tissue Punches

Frozen brains were mounted in optimum cutting temperature compound (OCT, VWR) and placed in isopentane on dry ice. Micropunches of the NAc and AMY were taken as previously described (Osterndorff-Kahanek et al. 2015). The following coordinates, anterior-posterior distance from bregma, were utilized: NAc (+1.8 mm to +0.6 mm); AMY (-0.9 mm to -1.8 mm).

2.III.e. RNA Isolation and Quantitative RT-PCR

Half of each frozen PFC and the tissue punches were used for RNA isolation using the MagMax-96 Total RNA Isolation Kit (Thermo Fisher Scientific Inc., Rockford, IL). The RNA yield was quantified on a NanoDrop 1000 spectrophotometer and assessed for quality on an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA). RNA was reverse transcribed into cDNA using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc.). cDNA was tested for genomic DNA contamination and showed at least a 10 Cq difference between the +RT

(reverse transcription) and –RT samples (Bustin et al. 2009). Applied Biosystems TaqMan® Gene Expression Assay (Thermo Fisher Scientific Inc.) primers were used (*specific assay IDs are shown in* **Table 2.2**). Quantitative PCR reactions were performed using SsoAdvanced™ Universal Probes Supermix (BioRad, Hercules, CA) in 10-µL reactions containing 18 ng of cDNA. All reactions were performed in triplicate and included a negative control. qPCR reactions were carried out using the CFX384 Real-Time System (Thermo Fisher Scientific Inc.). For the PFC, three reference genes (Gusb, Gapdh, and Hprt) were tested for each time point and the best two genes were selected based on normalization factors in the qbase+ software (Biogazelle, Gent, Belgium). The 0-hour samples were normalized to Gapdh and Hprt; the 24-hour samples were normalized to Gapdh and Gusb. The NAc and AMY micropunches were normalized only to Gapdh due to smaller quantities of cDNA. Relative quantification of mRNA levels was determined using the qbase+ software.

2.III.f. Protein Isolation and Western Blot Analysis

Half of each PFC was used for protein isolation. Tissue was homogenized in 200 µL of lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1% Triton-X-100, 1% sodium deoxycholic acid, 0.1% SDS, 1X Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific Inc.), centrifuged for 10 minutes at 10,000 x g, aliquotted, and frozen at -80°C. Protein concentrations were determined using the DC Protein Assay (Bio-Rad). Cell lysates (40 µg) were boiled for 5 minutes, run on 10% Mini-Protean TGX Precast Gels (Bio-Rad), and transferred to PVDF membranes using

semi-dry transfer. Membranes were blocked with 5% dried milk in TBST (Tris-buffered saline with 0.5% Tween-20) and incubated overnight at 4°C with primary antibody (**Table 2.3**). Membranes were washed with TBST and incubated with HRP-conjugated secondary antibodies in 5% dried milk in TBST. Bands were visualized using ECL (Pierce) and imaged on film and using G:BOX Chemi XX6 (Syngene, Cambridge, UK). Bands were quantified using ImageJ and normalized to GAPDH and β-actin. All blots were repeated at least once.

2.III.g. Immunohistochemistry

Brains were post-fixed for 24 hours in 4% PFA at 4°C, cryoprotected for 24 hours in 20% sucrose, and mounted in molds with optimal temperature compound (OTC). Frozen brains were sectioned coronally (20 μm thick) and placed free floating into PBS. Sections were permeabilized in optimized detergent (0.1% Triton-X-100 or 0.1% SDS) and blocked in 10% goat or donkey serum for 1 hour at room temperature. Sections were incubated with primary antibody overnight at 4°C (**Table 2.3**). The following day, sections were washed with PBS and incubated with secondary antibody for 2 hours at room temperature. Appropriate quality control staining was performed for labeling and secondary antibodies (**Fig. 2.7**). Sections were mounted on slides using Vectashield containing DAPI (Vector Labs, Burlingame, CA).

2.III.h. Microscopy

Quantification of immunopositive cells was performed using a Zeiss Axiovert 200M fluorescent light microscope (Zeiss, Thornwood, NY) equipped with an Axiocam b/w camera. Brain regions were identified using a mouse brain atlas as previously described (Zuloaga et al. 2014). Bilateral images of the PFC (distance from bregma: +2.8 mm to +2.24 mm), NAc (+1.10 mm to +0.8 mm), and AMY (-1.20 mm to -1.60 mm) were captured using a 20x objective. IRF3 and IKKε immunopositive cells were quantified bilaterally within fixed area frames: PFC (box, 645 am x 645 μm), NAc (circle, 575 μm diameter), and AMY (circle, 675 μm diameter), using the ImageJ plug-in ITCN (http://rsb.info.nih.gov/ij/plugins/itcn.html). Areas used for quantification are shown in **Figure 2.8**. We quantified immunoreactivity as the number of immunopositive cells per section divided by total cell count (i.e. DAPI). For all cell quantifications, cells were counted in both hemispheres for a given region and summed. Total cell counts for each animal were then averaged and are presented as % immunopositive cells per section.

2.III.i. Amlexanox Treatment

Mice consumed 15% ethanol for at least 8 weeks using the EOD-2BC paradigm described above. After this period ethanol consumption was measure for at least 4 days to ensure stable consumption. Ethanol intake was then measured after saline administration for 2 days and mice were grouped to provide similar levels of ethanol intake and preference based on the consumption during these 2 days. On day 4, mice were administered saline or Amlexanox once daily and results are presented as the average from 2-day periods of consecutive drinking using different bottle positions. Amlexanox

(100 mg/kg) was administered orally by gavage (p.o.). Amlexanox was purchased from Abcam (ab142825). Drug was prepared as suspensions in saline with 5-4 drops of Tween-80 and administered once daily in a volume of 0.05 ml/10g of body weight 30 minutes before drinking experiments. Saline containing 4-5 drops of Tween-80 was administered to control groups.

2.III.j. Amlexanox-Poly I:C Treatment

Mice were divided into four groups (n=10 per group) and were given an oral treatment (saline or Amlexanox 100 mg/kg) followed by an I.P. injection 30 minutes later (saline or Poly I:C 5mg/kg). Mice were sacrificed 3 hours after the I.P. injection and the PFC was fresh harvested as described above. RNA isolations and qPCR analysis were performed as described above.

2.III.k. Statistical Analysis

Results are reported as the mean ± SEM. Data were analyzed using GraphPad software (San Diego, CA) and 2-tailed Student's t-tests unless otherwise noted (unpaired parametric test, assumes populations have the same SD). Statistical outliers were identified using Grubb's test and removed from analysis. Consumption following Amlexanox or Saline treatment was analyzed using a 2- way analysis of variance (ANOVA) and Tukey's multiple comparisons test. Gene expression changes following Poly I:C and Amlexanox treatments were analyzed using a 1-way ANOVA and Tukey's multiple comparisons test.

2.IV. RESULTS

2.IV.a. Chronic ethanol consumption increases expression of Tlrs

To determine whether chronic ethanol consumption changes mRNA levels in the TLR signaling pathways in the PFC, qPCR analysis was performed 0 and 24 hours after ethanol removal (**Fig. 2.1**). Several TLRs are altered in other studies using different alcohol paradigms (Alfonso-Loeches et al. 2010; Crews et al. 2013; Whitman et al. 2013; Lippai et al. 2013). Consistent with previous studies, qPCR analysis confirmed that *Tlr2, Tlr3, Tlr4, and Cd14* (TLR co-receptor) are increased following voluntary ethanol consumption at the 24-hour time point (**Fig. 2.1a**). Although *Tlr2* and *Tlr4* have been implicated in alcohol action in more studies than *Tlr3* (G. Robinson et al. 2014), the largest fold-change (2.12) was observed for *Tlr3*. Like *Tlr3, Tlr7* is another endocytic TLR that recognizes viral RNA and leads to the production of Type I interferons, despite signaling through the MyD88-dependent pathway. *Tlr7* has been implicated in alcohol liver disease (Cha et al. 2012) and in the suppression of cytokines by acute ethanol exposure (Pruett et al. 2004). To determine whether *Tlr7* expression changed with chronic ethanol consumption, qPCR was performed at both time points. In contrast to the other TLRS, *Tlr7* expression increased 0 hours after ethanol removal.

2.IV.b. Chronic ethanol consumption increases MyD88 and TRIF-related mRNA levels in a time dependent manner

qPCR was then used to evaluate changes in components of the MyD88-dependent pathway, which is common to all TLRs except TLR3, and hypothesized to be affected by

chronic drinking. Only modest increases were observed for *Myd88* and *Irak4* at 0 hours, and all MyD88 pathway components were unchanged at 24 hours (**Fig. 2.1b**). Investigation of the TRIF-dependent pathway revealed an increase in *Ikke* at 0 hours and an increase in *Trif* and *Irf3* 24 hours after ethanol removal (**Fig. 2.1c**). *Tbk1* mRNA expression was unchanged. These results suggest that chronic ethanol consumption mainly changes expression of TRIF-dependent pathway mRNA. There are also molecules that are common to both the MyD88 and TRIF-dependent pathways. *Traf3* and *Irf7* are involved in both TRIF-dependent signaling and TLR7 signaling through the MyD88-dependent pathway while *Traf6* is involved in all MyD88- and TRIF-dependent signaling. Measurement of these mRNAs using qPCR revealed that *Traf3 and Traf6* were unchanged at both time points, but *Irf7* expression increased at 0 hours (**Fig. 2.1d**).

2.IV.c. Chronic ethanol consumption increases Il1b and Cxcl10 mRNA immediately after ethanol removal and Il6 24 hours after ethanol removal

TLR pathway activation leads to transcription of cytokines, chemokines, and interferons and several cytokine mRNAs have been previously linked to alcohol exposure (*Il1b, Tnfa, and Il6)* (Zou & Crews 2010; Alfonso-Loeches et al. 2010; Whitman et al. 2013; Lippai et al. 2013). Expression of these cytokines were measured to evaluate activation of the MyD88-dependent pathway. In addition, transcriptional outputs of the TRIF-dependent pathway (*Ifnb*, *Ccl5*, and *Cxcl10*) were measured, however, only *Cxcl10* was expressed at high enough levels to be quantified. Expression of *Il1b* and *Cxcl10* mRNA increased at 0 hours while *Il6* expression increased 24 hours after ethanol

removal (**Fig. 2.1e**). *Il1b* transcription is primarily induced by MyD88-dependent signaling, while *Cxcl10* is mostly induced by TRIF-dependent signaling and *Il6* requires both pathways (Hirotani et al. 2005). These data suggest that both pathways may be activated in response to chronic ethanol exposure.

2.IV.d. Chronic ethanol consumption increases a microglial, but not an astrocyte, marker at 24 hours

Several studies have shown that glial activation is an important component of ethanol-induced neuroimmune signaling (He & Crews 2008; Lippai et al. 2013; Fernandez-Lizarbe et al. 2009). Microglia and astrocytes respond to pathogens and danger signals (i.e. HMGB1) by releasing cytokines, which can then further activate glia (Crews & Vetreno 2016). To determine the impact of chronic voluntary ethanol consumption on glial activation, we measured the astrocyte marker *Gfap*, the microglial marker *Cd11b*, and the activated microglial marker *Cd68*. *Cd68* expression decreased at 0 hours while *Cd11b* mRNA increased 24 hours after ethanol removal (**Fig. 2.1f**). No additional changes in glial expression were observed.

2.IV.e. Chronic ethanol consumption did not change protein levels in the PFC

After gene expression changes were observed for TLRs and TRIF-dependent pathway transcripts, protein levels were measured from the same PFC tissue using western blots. No significant differences were seen in any of the proteins measured (**Fig.**

2.9). Due to the small fold-changes it is possible that western blots are not sensitive enough to detect protein level changes.

2.IV.f. Chronic ethanol consumption leads to increased IKKε and IRF3 immunopositive cells in the PFC and NAc

Immunohistochemistry, which may be a more sensitive measurement compared to western blots, was used to determine the number of IKKε and IRF3 immunopositive cells (**Fig. 2.2**). In the PFC, there was a significant increase in the percentage of IKKε immunopositive cells at 0 hours and a significant increase in the percentage of both IKKε and IRF3 immunopositive cells 24 hours after ethanol removal (**Fig. 2.2a**). The increases were larger at the 24-hour time point, which is consistent with the qPCR data. It is interesting to note that changes in both mRNA and IKKε protein are seen at 0 hours. In addition to the PFC, immunopositive cells were measured in the NAc and AMY. The NAc showed similar changes to the PFC, with a small increase in IKKε and IRF3 at 0 hours and larger increases in both IKKε and IRF3 at 24 hours (**Fig. 2.2b**). No changes were found in the AMY at 0 hours, but there was a decrease in IKKε and an increase in IRF3 at 24 hours (**Fig. 2.2c**). These results suggest that components of the TRIF signaling pathway are increased on the protein level by chronic ethanol in a brain region-specific manner.

2.IV.g. Chronic ethanol consumption leads to increased Tlr3 mRNA in the NAc and decreased Tlr mRNA in the AMY

Because changes in the percentage of immunopositive cells in the NAc and AMY were observed, mRNA levels in these brain regions were measured from micropunch samples (**Fig. 2.3**). Due to limited sample, only transcripts that changed in the PFC were measured. In the NAc, there was an increase in *Tlr3* mRNA at the 24-hour time point while there was a decrease in *Tlr3* and *Tlr4* at 24 hours in the AMY (**Fig. 2.3a**). Despite changes in immunopositive cells, there were no changes in expression in the TRIF-dependent pathway components at either time point in either brain region (**Fig. 2.3b**). The AMY showed a decrease in *Cxcl10* at the 24-hour time point, consistent with the decreased expression of *Tlr3/4* and IKKε immunopositive cells seen in that brain region (**Fig. 2.3c**). Like the PFC, both the NAc and AMY showed an increase in *Il1b* expression at the 0-hour time point (**Fig. 2.3c**). This early increase in *Il1b* has also been observed in the hippocampus and VTA (data not shown), suggesting a brain-wide increase in *Il1b* immediately after ethanol removal.

2.IV.h. Inhibitor of TRIF-dependent pathway decreases ethanol consumption

To evaluate whether changes in TRIF-dependent signaling may regulate ethanol consumption, mice were treated with the dual IKKε/TBK1 inhibitor Amlexanox. Amlexanox decreases kinase activity of IKKε and TBK1 as well as phosphorylation of IRF3 in response to the TLR3 ligand polyinosinic:polycytidylic acid (Poly I:C) (Reilly et al. 2013). At a dose of 100 mg/kg, Amlexanox reduced ethanol consumption and preference in mice undergoing a chronic EOD-2BC paradigm but did not significantly

change total consumption (**Fig. 2.4**). These data suggest a role for activation of the TRIF-dependent pathway in the regulation of ethanol consumption.

2.IV.i. Amlexanox reduces Poly I:C induced increase in Cxcl10 expression

Amlexanox decreases the Poly I:C response *in vitro* and decreases high-fat diet induced expression of *Cxcl10* in liver, but there is no evidence that it inhibits the TRIF-dependent pathway in brain. To address this, we measured *Cxcl10* expression in the PFC of mice that were treated with saline only, Amlexanox only, the TLR3 ligand Poly I:C, and both Poly I:C and Amlexanox. Administration of Poly I:C resulted in a ~2,000-fold increase in *Cxcl10* expression, however administration of Poly I:C with Amlexanox reduces the Poly I:C-induced increase by 40% (**Fig. 2.11**).

2.V. DISCUSSION

Emerging evidence supports the role of neuroinflammatory mechanisms in alcohol dependence and alcohol-induced brain damage. Our group and others have shown that ethanol upregulates inflammatory mediators in the brain, which activate neuroimmune pathways (Blednov, Ponomarev, et al. 2011; Crews et al. 2013). Studies have focused on TLR4 because of its role in producing proinflammatory cytokines that can promote neuroinflammatory-dependent brain damage (Alfonso-Loeches et al. 2010; G. Robinson et al. 2014). Given that MyD88 is the adapter protein for TLR4 (and all other TLRs except TLR3), we hypothesized that chronic ethanol drinking would lead to increased expression of the MyD88-dependent pathway. However, our findings indicate

that EOD ethanol consumption primarily increases expression of *Tlr3* and components of the TRIF-dependent pathway (**Table 2.4**). We observed upregulation of TRIF signaling components at both the mRNA and protein levels as well as increased expression of *Cxcl10*, a transcriptional output of TRIF pathway activation. The TRIF-dependent pathway changes appear to be both brain region- and time-specific, with the greatest increase in signaling components observed in the PFC 24 hours after the last ethanol exposure and an increase in transcriptional outputs at 0 hours (see **Table 2.1**). Moreover, we provide evidence that the TRIF-dependent pathway may regulate drinking by showing that a IKKε/TBK1 inhibitor decreases ethanol consumption. A potential role for the TRIF-dependent pathway in ethanol consumption points to the utility of drugs that target this system for treating AUDs.

Chronic alcohol abuse encompasses a relapsing cycle of intoxication, withdrawal, and craving resulting in aberrant neuroplasticity in corticolimbic structures (e.g. PFC), mesolimbic (e.g. NAc), and the extended AMY circuit. Expression changes in immune-related genes in the PFC, NAc, and AMY following chronic intermittent ethanol treatment showed little overlap between brain regions, suggesting that there are region-specific differences in immune signaling (Osterndorff-Kahanek et al. 2015). Our results also support brain region-specific differences in neuroinflammatory signaling. The PFC showed the largest increase in *Tlr* mRNA expression and in TRIF-related expression of mRNA and protein. Similar changes in protein were found in the NAc, but only *Tlr3* mRNA increased in this region. This could be due to differences in temporal signaling events in these brain regions, suggesting a dominance of corticolimbic structures during

craving. It is also possible that TLR3 is the predominant subtype altered in the NAc, while TLR2, TLR3 and TLR4 all increase in the PFC. Interestingly, the AMY showed decreased *Tlr3, Tlr4, and Cxcl10* mRNA levels and decreased IKKε protein at 24 hours. However, increased IRF3 protein expression was observed in the AMY at 24 hours, potentially due to decreased TLR signaling and compensatory increased IRF3 signaling. Although Tlr and TRIF-dependent changes in the AMY were the opposite of those seen in the PFC and NAc, all three brain regions showed an increase in *Il1b* at the 0-hour time point suggesting that there may be distinct signaling events occurring. This increase in *Il1b* is noteworthy because of the involvement of the IL-1 system with GABAergic transmission and evidence that IL1 is also involved in disorders, like depression, that have high comorbidity with AUDs (Bajo, Herman, et al. 2015; Bajo, Varodayan, et al. 2015; Barnes et al. 2017). Gene expression studies support the idea that the PFC, NAc and AMY show both common and unique gene expression changes, with modules of co-expressing genes sometimes changing in the opposite direction in different brain regions (Osterndorff-Kahanek et al. 2015). Our inhibitor (Amlexanox) results show that global inhibition of the TRIF pathway reduces ethanol consumption. It is important to note that inhibition of TLR4 signaling does not reduce alcohol consumption emphasizing the importance of the TLR3/TRIF pathway (Harris et al. 2017; Blednov et al. 2017).

The EOD drinking paradigm produces escalation of intake and maintains high alcohol consumption on drinking days (Crabbe et al. 2012). The 0-hour and 24-hour time points were selected to compare neuroimmune responses at different stages during chronic alcohol consumption (i.e. craving vs. bingeing). The 0-hour time point assesses

immune activation during the last ethanol exposure while ethanol is still present, albeit in low levels; whereas, the 24-hour time point represents the post-withdrawal stage when the mice are anticipating the availability of alcohol. We observed effects of alcohol consumption that are dependent on the time after withdrawal with more MyD88-dependent pathway changes at the 0-hour time point while most of the TLR and TRIF-dependent pathway changes are at the 24-hour time point, consistent with the idea that the MyD88-dependent pathway is activated earlier than the TRIF-dependent pathway (Kawai & Akira 2007). In addition, we saw increased expression of *Cxcl10*, a transcriptional output of TRIF-signaling, at the 0-hour time point. This is consistent with the idea that the pathway is being activated at 24 hours leading to increased transcription observed at 0 hours. Because we observed the largest increase in TRIF-dependent components after 24 hours, we suggest that this pathway may be involved in the post-withdrawal craving stage. Amlexanox reduced ethanol consumption when administered at this 24-hour time point (right before drinking was re-instated), further suggesting that TRIF signaling is involved in craving. There are some timing inconsistencies, such as *Ikke* increasing at the 0-hour time point and not the 24-hour time point or *Il6* and *Il1b* changing at different time points. TLR signaling is complex and involves many levels of regulation, including negative and positive feedback as well as components that are common to multiple pathways (Medzhitov & Horng 2009). It is likely that any perturbation of this pathway could impact expression of other signaling molecules involved, and that the timing of these changes would depend on the speed and level of regulation.

Many of the TLR pathway components measured here have been studied following other methods of alcohol exposure. *Il1b* increased in mouse cortex after an ethanol-based diet and *Il6* increased in mouse cerebellum after binge ethanol exposure (Whitman et al. 2013; Kane et al. 2013). *Tlr2* and *Tlr4* have been shown to increase after various drinking paradigms and *Tlr3* was increased following chronic intragastric ethanol exposure (Crews et al. 2013; Whitman et al. 2013; Lippai et al. 2013). In vitro studies in microglia and astrocytes have shown that ethanol can activate both the MyD88-dependent and TRIF-dependent pathway, consistent with our detection of changes in components of these pathways *in vivo* (Fernandez-Lizarbe et al. 2009; Pascual-Lucas et al. 2014). In contrast to our results, some studies reported increases in MyD88 and TLR protein, *Gfap* mRNA, TNF-α (mRNA and protein), and IL-1β protein (Alfonso-Loeches et al. 2010; Crews et al. 2013; Whitman et al. 2013; Lippai et al. 2013). These differences are likely due to the differing alcohol consumption paradigms, which are known to produce distinct effects on gene expression (Osterndorff-Kahanek et al. 2015; Osterndorff-Kahanek et al. 2013). Our current results represent the expression changes following chronic voluntary ethanol consumption and the differences that are manifested immediately after consumption and after withdrawal.

A caveat of this study is that we were unable to show increased phosphorylation of IRF3 as a measure of pathway activation. We attempted to measure phosphorylated IRF3 and interferon expression, but were unable to detect them at high enough levels in brain (**Fig. 2.10**). However, we were able to measure the interferon inducible gene *Cxcl10*, which is transcribed in response to TRIF-pathway activation (Hirotani et al.

2005; Weighardt et al. 2004). Our data showing that the TLR3 ligand Poly I:C leads to increased *Cxcl10* expression in the PFC, further supports this chemokine as a measure of pathway activation. Due to the transient nature of phosphorylation states, changes in transcriptional outputs is a more reliable measure of pathway activation. The increased *Cxcl10* expression in the PFC at the 0-hour time point supports the idea that the TRIF-dependent pathway is being activated in response to chronic ethanol. *Cxcl10*/IP-10 is a chemokine that is responsible for recruiting cells that express CXCR3 (microglia, dendritic cells, and T lymphocytes) and has roles in regulating apoptosis, cell growth and proliferation, and synaptic activity (Gruol 2016; Mingli Liu et al. 2011). Increased IP-10 therefore could increase the immune response to ethanol as well as contribute to the alcohol-induced damage or changes in neuronal activity. In addition, the fact that an inhibitor of this pathway decreases drinking and partially rescues Poly I:C-induced *Cxcl10* increases, further supports the idea that TRIF-pathway activation is involved in the ethanol response as well as regulation of consumption.

The fold changes reported here are relatively small and it is important to consider that there may be a dilution effect when examining heterogeneous cell populations. If immune signaling molecules are preferentially expressed in glial cells, studying the entire brain region would minimize any cell type-specific changes that may be present (Mayfield et al. 2013). We have observed greater differences in gene expression in more homogenous cell fractions, which can reveal discrete, localized changes (Most, Workman, et al. 2014). In addition, the drinking paradigm we used here is voluntary and

does not produce as extreme changes as those seen with high dose paradigms. For example, 5 g/kg intragastric ethanol daily for 10 days (a binge model) produced increases in Tlr mRNA that ranged from 1.5 -2.5 fold (Crews et al. 2013).

In summary, voluntary chronic ethanol consumption increases expression of brain *Tlrs*, particularly *Tlr3* and components of the TRIF-dependent pathway, 24 hours after ethanol exposure. These changes could promote ethanol consumption and dependence, supported by the fact that a TRIF-pathway inhibitor decreased ethanol consumption.

2.VI. Acknowledgements

The authors thank Olga Ponomareva, Jillian Benavidez, Mendy Black, and Adriana DaCosta their technical assistance and Jody Mayfield for her manuscript edits. This work was supported by the NIAAA grants AA012404, AA024654, AA013520, AA006399, and AA020683. The authors declare no conflict of interest regarding this research.

2.VII. Author Contributions

GMM, YAB, and RAH were responsible for the study concept and design. YAB provided animals, YAB and GMM performed animal experiments. GMM and CRB collected tissue and ASW performed tissue punches. GMM and CRB performed qPCR analysis, GMM performed western blots, and ASW performed immunohistochemistry. GMM drafted the manuscript, ASW, CRB, and RAH provided manuscript revisions.

GMM and RAH performed data analysis and interpretation. All authors critically reviewed content and approved final version for publication.

2. VIII. FIGURES

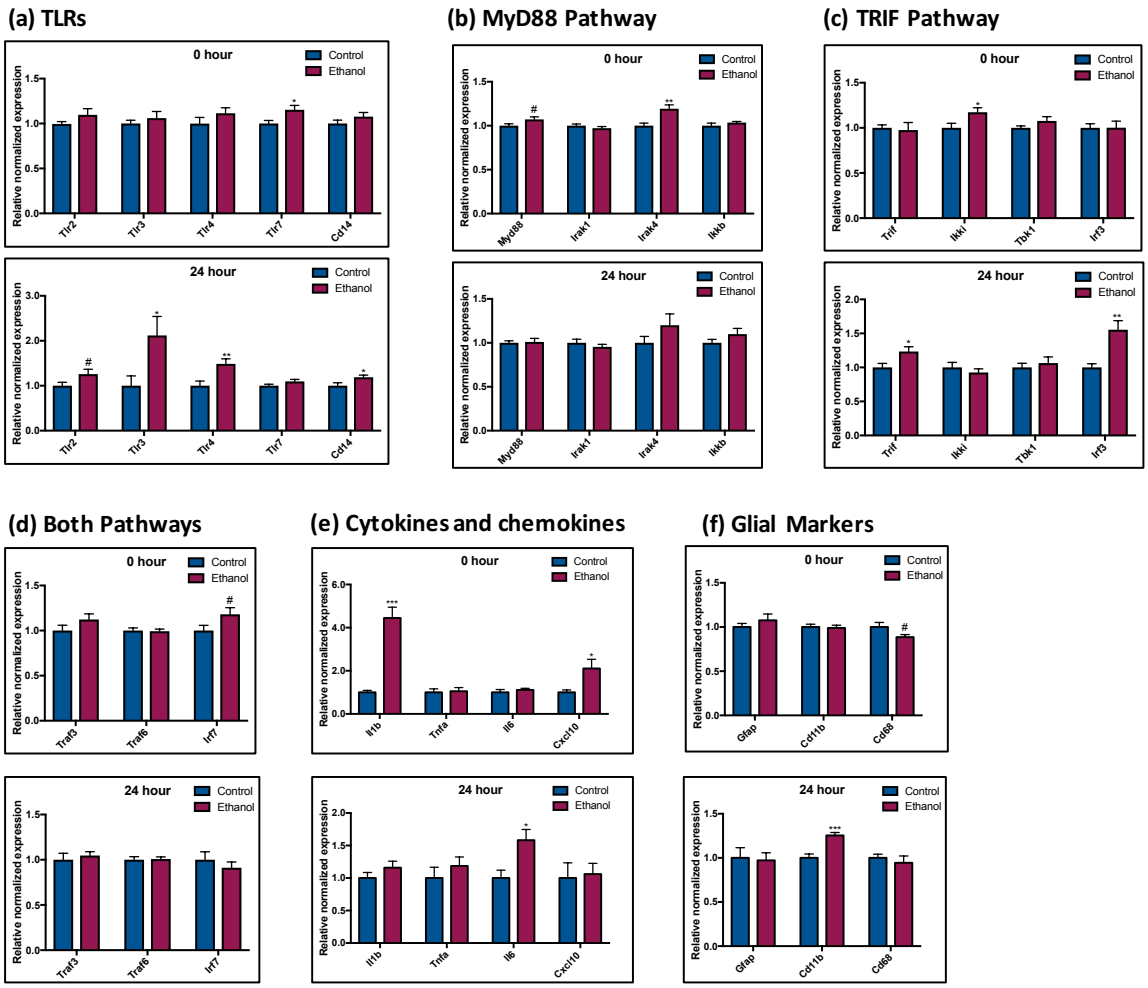


Figure 2.1: Ethanol induced mRNA changes in the PFC

Figure 2.1: Continued

RT-qPCR analysis of the PFC 0 or 24 hours after ethanol removal (n=10/group). (a) The 0-hour group showed increased *Tlr7* expression while the 24-hour ethanol group showed increased *Tlr2, Tlr3, Tlr4* and *Cd14* mRNA expression. (b) The 0-hour ethanol group showed increased *Myd88* and *Irak4* expression. (c) Increased *Ikki* expression was observed in the 0-hour group, while increased *Trif* and *Irf3* expression was found in the 24-hour ethanol group. (d) The 0-hour group showed increased *Irf7* expression with ethanol. (e) The 0-hour ethanol group showed increased *Il1b* and *Cxcl10* expression while the 24-hour ethanol group showed increased *Il6* expression. (e) The 24-hour ethanol group showed increased expression of *Cd11b*, a microglial marker. All values are expressed as fold change over control ± SEM. # $p < 0.05$ compared to control, 1-tailed t-test, * $p < 0.05$ compared to control, 2- tailed t-test; ** $p < 0.01$ compared to control, 2-tailed t-test; *** $p < 0.001$ compared to control, 2-tailed t-test.

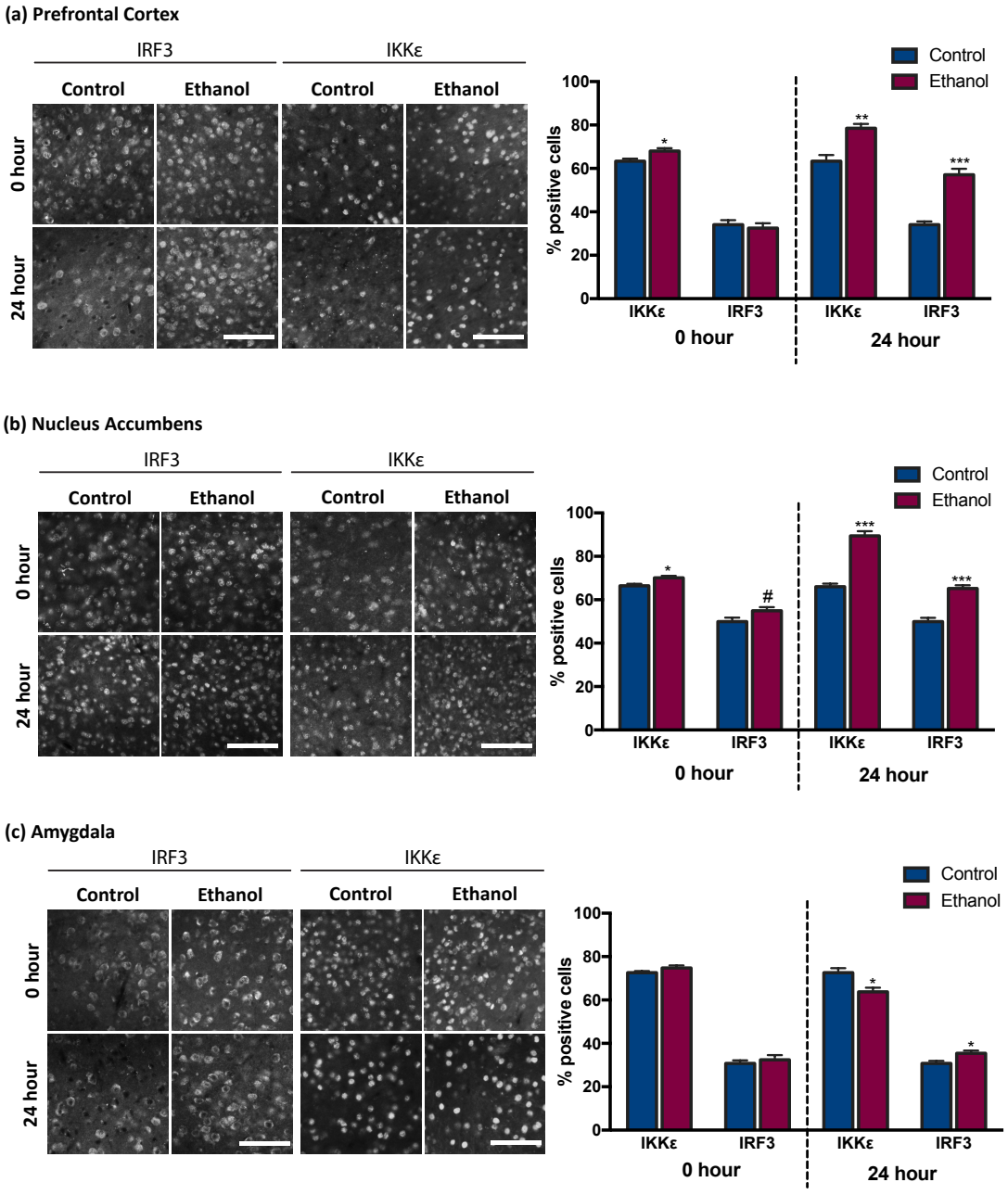


Figure 2.2: Immunohistochemistry of IRF3 and IKKε after ethanol

Figure 2.2: Continued

Immunohistochemistry for IRF3 and IKKε 0 and 24 hours after ethanol removal. Representative images are shown from one control and one ethanol sample. Graphs show quantification from all samples (n=5/ group) and values represent the average ± SEM percent of cells positive for either IKKε or IRF3 over the total number of DAPI positive cells. Scale bar for PFC and NAc = 50 μM and for AMY = 100 μM. (a) In the PFC there is an increase in the percentage of IKKε positive cells at 0 hours and of both IKKε and IRF3 positive cells at 24 hours (b) In the NAc there is increase in the percentage both IKKε and IRF3 positive cells at 0 and 24 hours (c) in the AMY there are no changes at 0 hours, and at 24 hours there is a decrease in IKKε positive cells and an increase in IRF3 positive cells. # $p < 0.05$, 1-tailed t-test, * $p < 0.05$ compared to control, 2- tailed t-test; ** $p < 0.01$ compared to control, 2-tailed t-test; *** $p < 0.001$ compared to control, 2-tailed t-test.

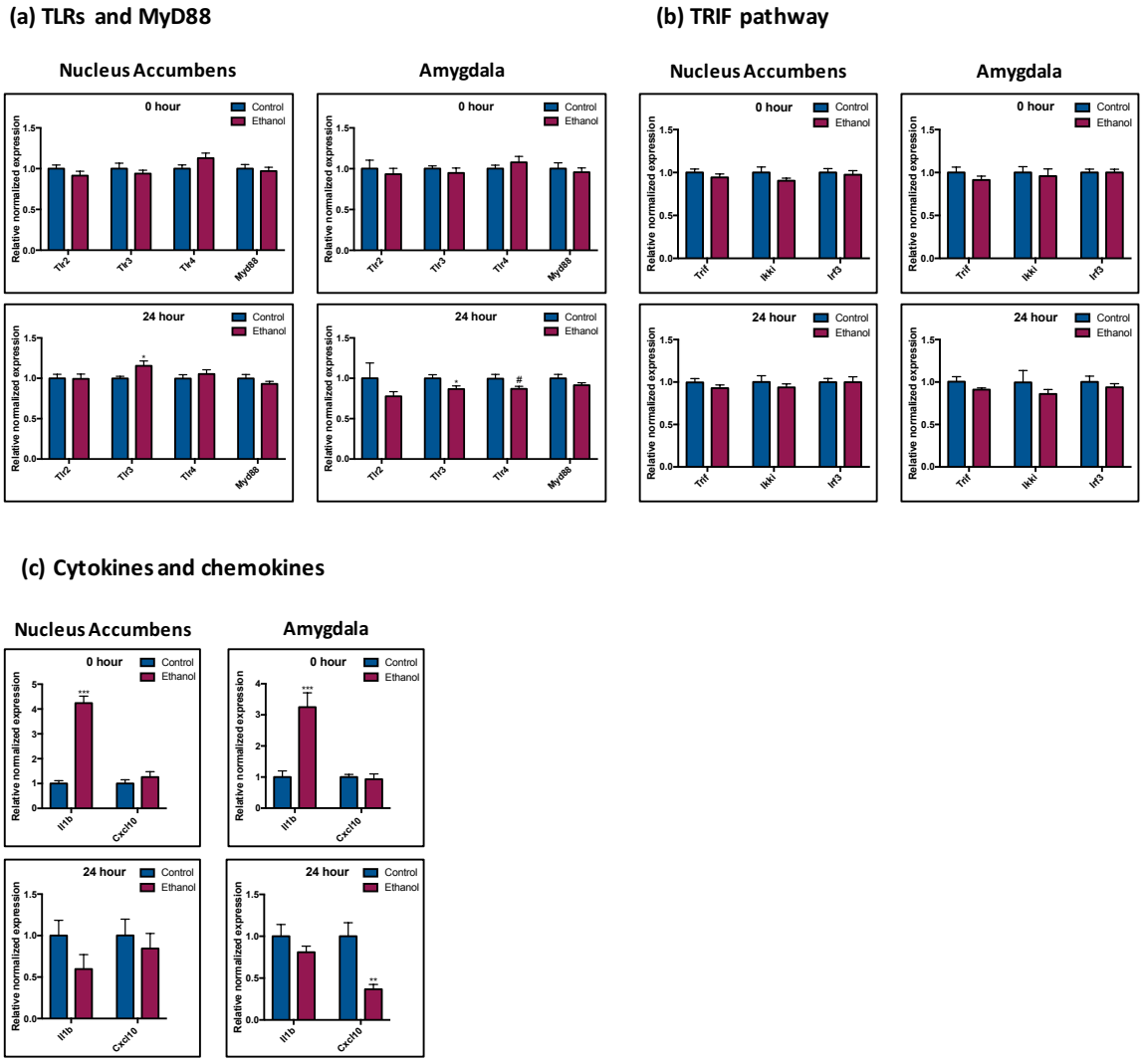


Figure 2.3: Ethanol induced mRNA changes in the Nucleus Accumbens and Amygdala

RT-qPCR analysis from the NAc and AMY 0 and 24 hours after ethanol removal (for the 0-hour time point n=10/ group and for the 24-hour time point n= 10 for the control group and n= 9 ethanol group). (a) The NAc shows increased *Tlr3* mRNA levels while the AMY shows decreased *Tlr3* and *Tlr4* at 24 hours. (b) No changes were seen in the TRIF pathway genes. (c) Both the NAc and AMY showed increased *Il1b* expression at 0 hours and the AMY showed decreased *Cxcl10* expression at 24 hours. All values are expressed as fold change over control ± SEM. # $p < 0.05$ compared to control, 1-tailed t-test; * $p < 0.05$ compared to control, 2- tailed t-test; ** $p < 0.01$ compared to control, 2-tailed t-test, *** $p < 0.001$ compared to control, 2-tailed t-test.

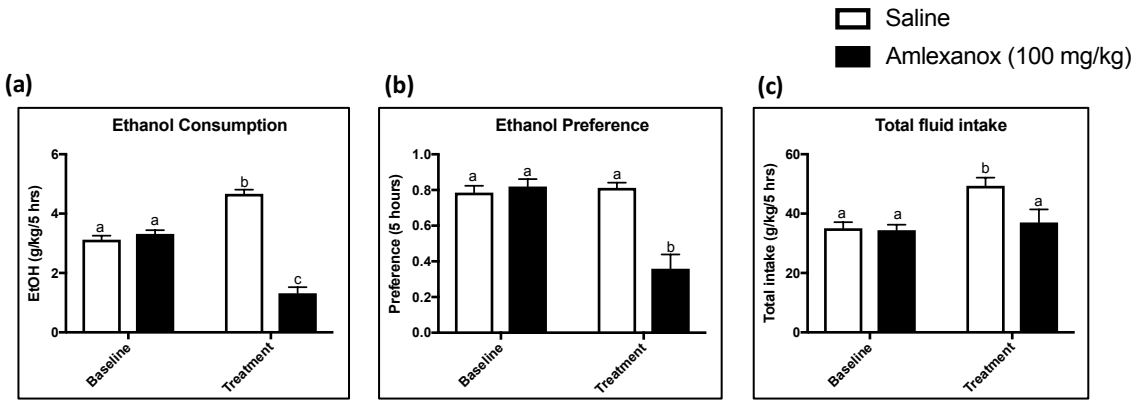


Figure 2.4: Effect of Amlexanox on ethanol consumption and preference

Measures for Saline and Amlexanox (100 mg/kg) treatment groups at baseline (both groups received saline injections) and with treatment. All measurements are taken 5 hours following injection. (a) The Amlexanox group shows decreased ethanol consumption compared to baseline and compared to the saline group. (b) The Amlexanox group shows decreased preference for ethanol compared to baseline and saline. (c) The Amlexanox group shows no change in total fluid intake compared to when it was treated with saline. Bars labeled with the same letter are not statistically different while bars with different letters are (2-way ANOVA with Tukey's multiple comparisons test, n=11 per group).

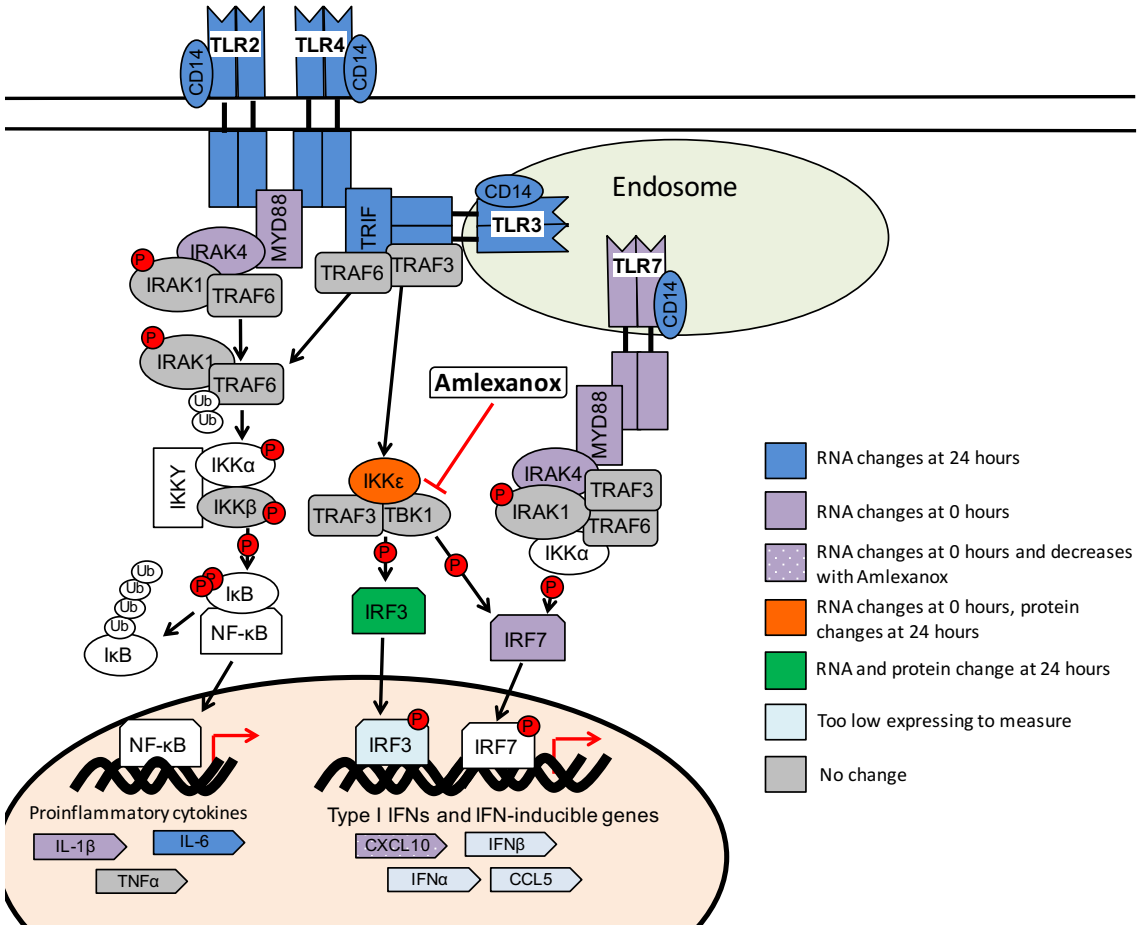


Figure 2.5: Summary of Ethanol changes in the PFC

Schematic of the TLR signaling pathways in the PFC. Dark blue represents RNA changes at 24 hours, purple represents RNA changes at 0 hours, black dots indicate that Poly I:C induced mRNA was decreased with Amlexanox, checkered hatching represents protein increases at 0 and 24 hours while diagonal hatching represents protein increases at 24 hours. Grey represents no change and white indicates that the pathway component was not measured in this study. Light blue means that expression was too low to measure in this study.

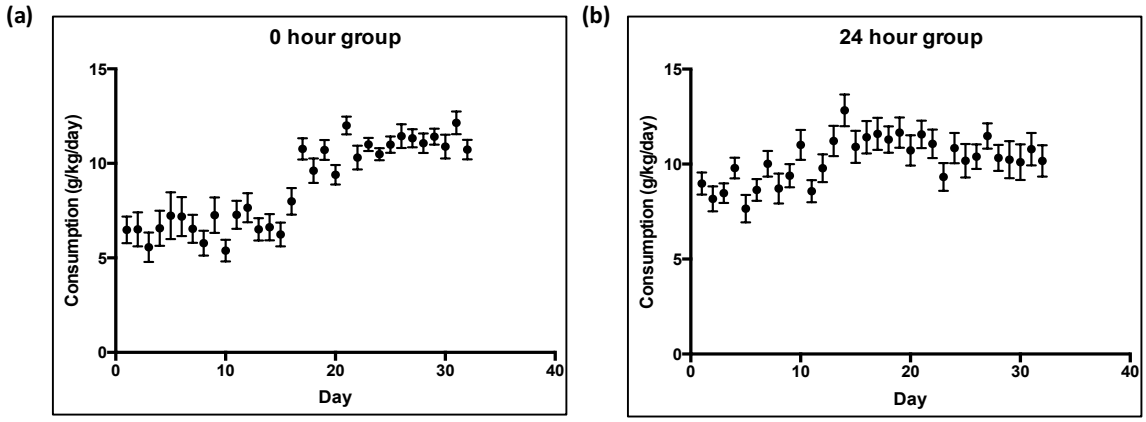


Figure 2.6: Ethanol Consumption

Average consumption shown as mean (g/kg/day) ± SEM for all animals over the course of the 15% ethanol EOD- 2BC drinking experiment (n= 15 for each time point). The x-axis represents days where ethanol was present.

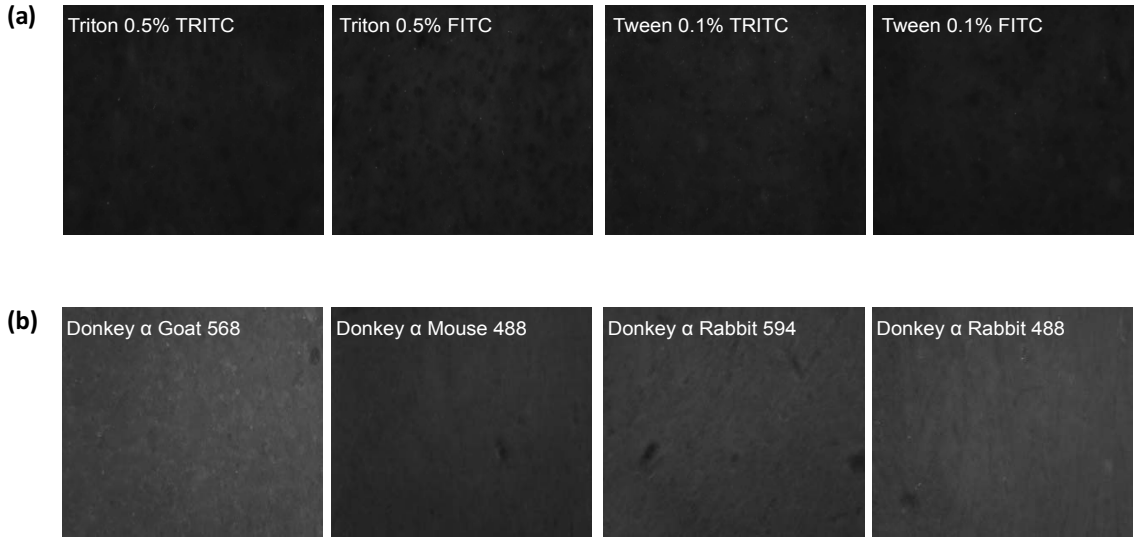


Figure 2.7: Control images for immunohistochemistry

Quality Controls for Immunohistochemical Quantification. (a) Labeling staining controls for all staining conditionings in nucleus accumbens of a C57Bl6/J male mouse--primary antibody and secondary antibody omitted, 10% donkey serum only. Image taken on fluorescent microscope (20x).(b) Secondary controls for all secondaries in nucleus accumbens of a C57Bl6/J male mouse--primary antibody omitted, 10% donkey serum and appropriate secondary. Image taken on a fluorescent microscope (20x).

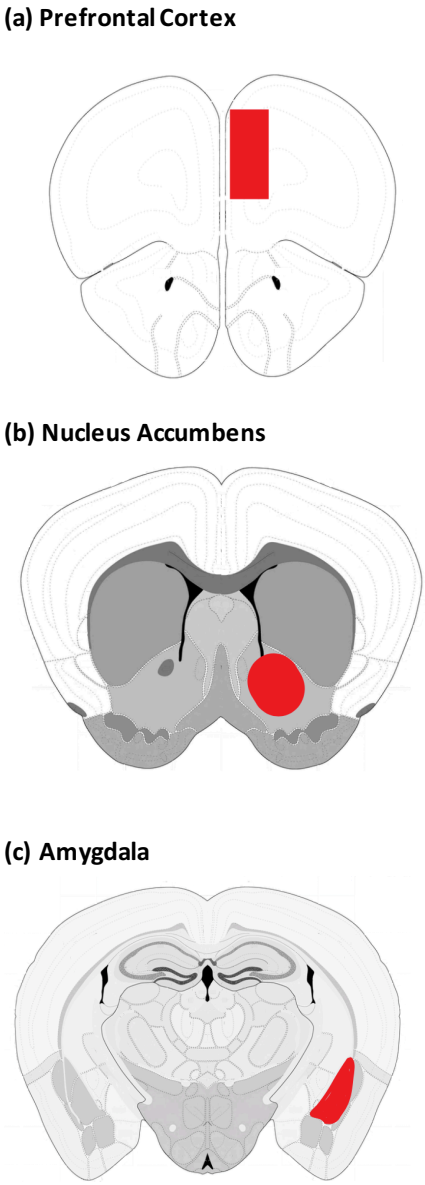


Figure 2.8: Brain regions for immunohistochemistry

Brain regions used for immunohistochemistry counts: (a) PFC, (b) NAc and (c) AMY.

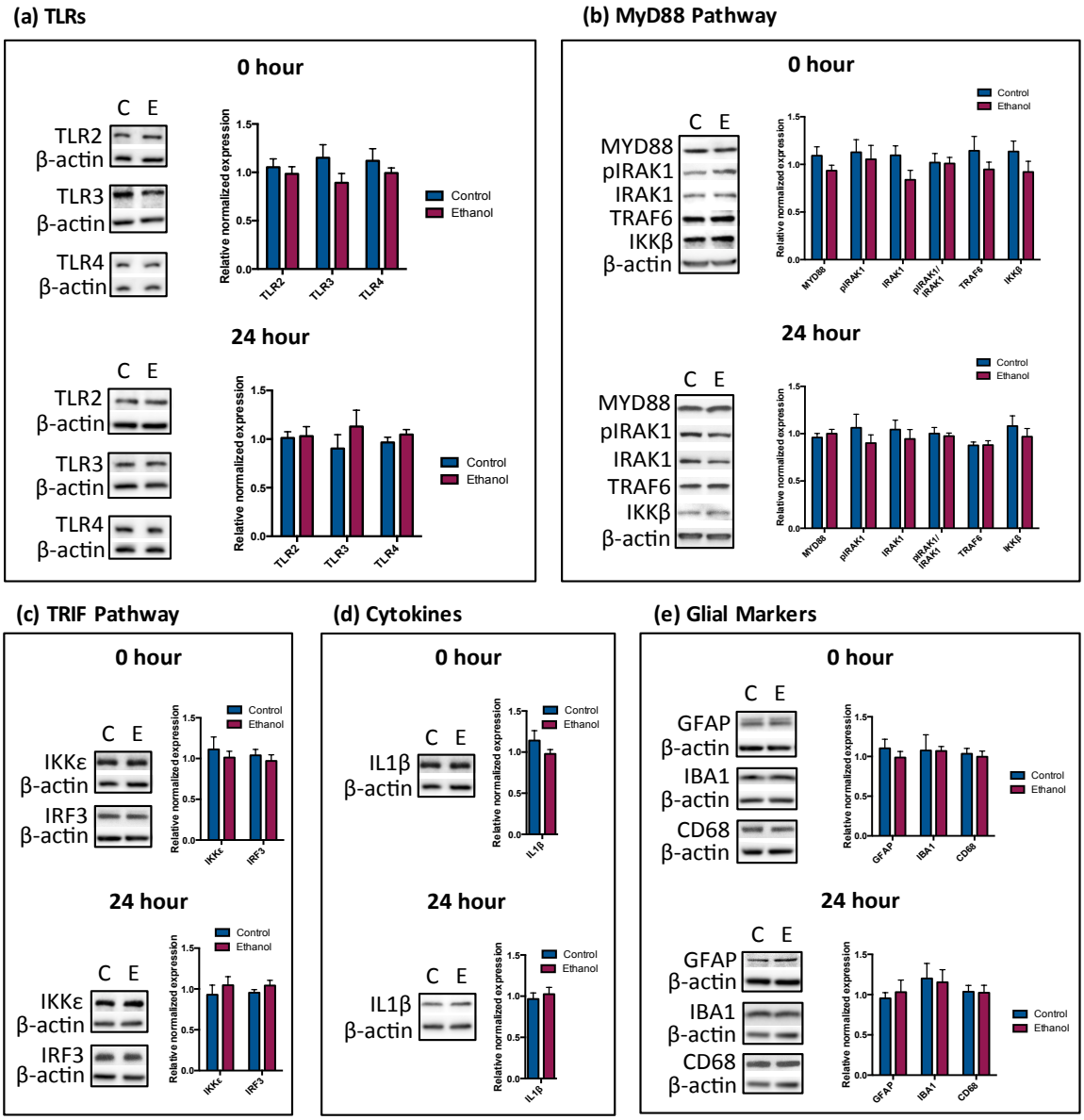


Figure 2.9: Western blots

Western blot analysis of the PFC 0 and 24 hours after ethanol removal. Representative blots are shown from one control (C) and one ethanol (E) sample. Graphs show quantification from a complete blot (n=10/group). Protein levels were normalized to β-actin and GAPDH. No differences were seen in (a) TLR protein levels, (b) MYD88 pathway protein levels, (c) TRIF pathway protein levels, (d) Cytokines protein levels, or (e) Glial marker protein levels. All values are expressed as relative protein expression (normalized to β-actin) ± SEM. Statistics were performed using a 2-tailed t-test.

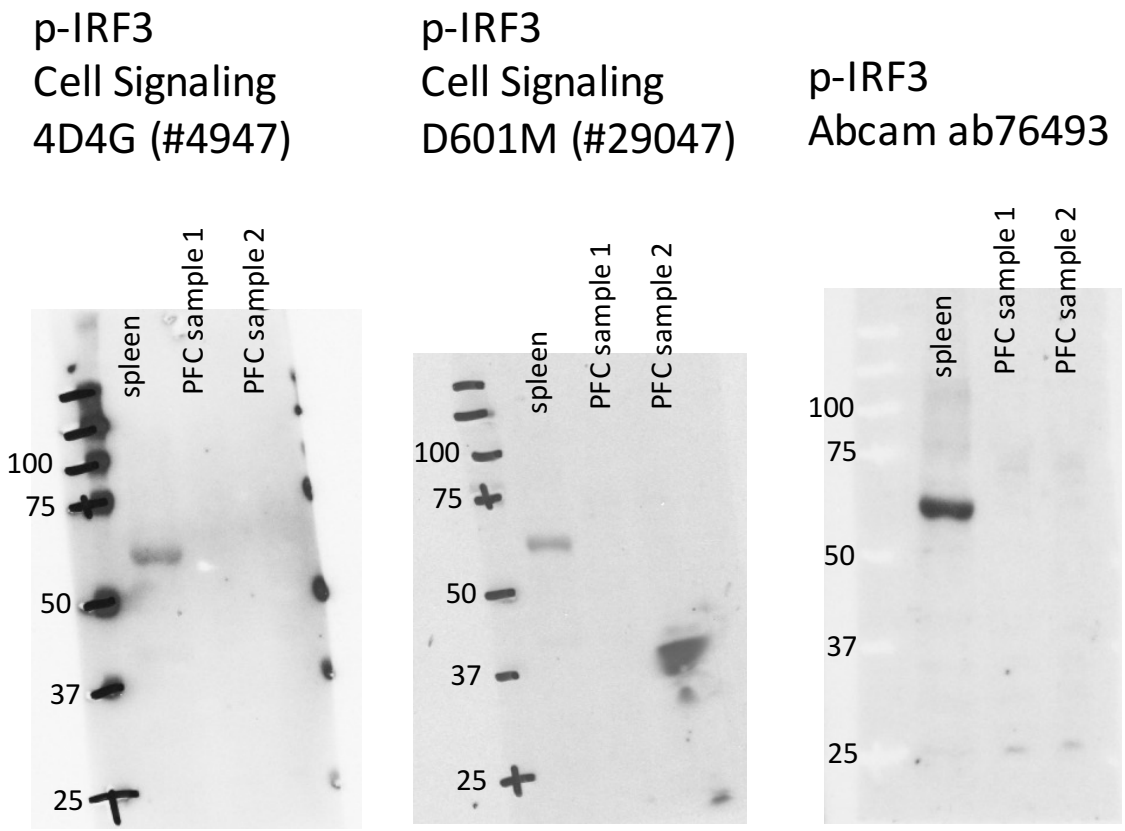


Figure 2.10: Western blot tests for p-IRF3

Western blots using 3 commercially available pIRF3 antibodies showing that signal is not able to be detected in the PFC despite being detected in the spleen.

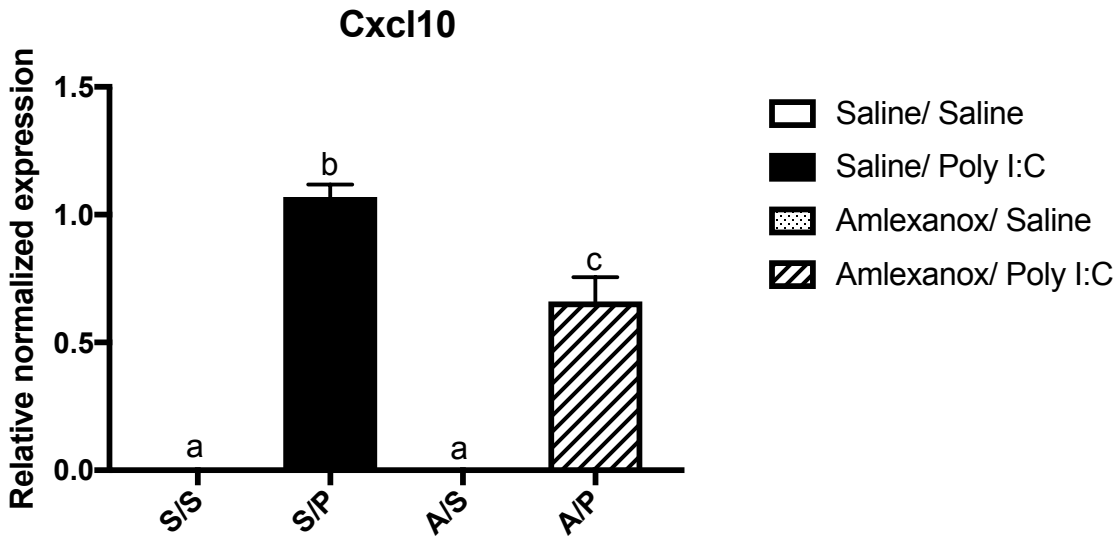


Figure 2.11: Amlexanox inhibits Poly I:C response in PFC

Cxcl10 qPCR analysis of the PFC after saline oral and saline I.P injection (S/S), saline oral and Poly I:C (5mg/kg) I.P. injection (S/P), Amlexanox oral (100 mg/kg) and saline I.P. injection (A/S), and Amlexanox oral (100 mg/kg) with Poly I:C (5 mg/kg) I.P. injection. Poly I:C significantly increases expression of *Cxcl10* mRNA while Amlexanox significantly decreases Poly I:C induced expression. Different letters indicate significantly different means (ANOVA with Tukey's multiple comparisons test, $p < .05$).

2.IX. TABLES

Sample #	mg/dL
1	13.3
2	10.1
3	12.2
4	5.6
5	9.7
7	11.0
8	7.8
9	15.1
10	11.1
11	18.2
13	18.7
14	7.0
15	15.6
16	5.3
17	8.2

Table 2.1: Blood alcohol content measurements

Blood alcohol concentration (mg/dL) at the 0-hour time point. Values are an average of two reads.

Gene Name	Gene Expression Assay
Cd14	Mm00438094_g1
Cd68	Mm03047343_m1
Cxcl10	Mm00445235_m1
Gapdh	Mm99999915_g1
Gfap	Mm01253033_m1
Gusb	Mm03003537_s1
Hprt	Mm01545399_m1
Ikbkb (Ikkb)	Mm01222247_m1
Ikbke (Ikki)	Mm00444862_m1
Il1b	Mm00434228_m1
Il6	Mm00446190_m1
Irak1	Mm01193538_m1
Irak4	Mm00459443_m1
Irf3	Mm00516784_m1
Irf7	Mm00516793_g1
Itgam (Cd11b)	Mm00434455_m1
Myd88	Mm00440338_m1
Tbk1	Mm00451150_m1
Ticam1 (Trif)	Mm00844508_s1
Tlr2	Mm00442346_m1
Tlr3	Mm01207404_m1
Tlr4	Mm00445273_m1
Tlr7	Mm00446590_m1
Tnf	Mm00443258_m1
Traf3	Mm00495752_m1
Traf6	Mm00493836_m1

Table 2.2: Taqman Assay IDs

Taqman® Gene Expression Assay IDs used for RT-qPCR

(a) Primary antibodies used for western blots

Antibody	Manufacturer	Catalog #	Host	Dilution	Diluant
β-Actin	Sigma	A1978	Mouse	1:10,000	5% NFDM
CD68	Abcam	ab31630	Mouse	1:1000	5% NFDM
GAPDH (FL-335)	Santa Cruz	sc-25778	Rabbit	1:5000	5% NFDM
GFAP	Invitrogen	18-0063	Rabbit	1:5000	5% NFDM
Iba1	Wako	019-19741	Rabbit	1:1000	5% NFDM
IKKi (M-17)	Santa Cruz	sc-5693	Goat	1:200	5% NFDM
IKKβ (P-20)	Santa Cruz	sc-34673	Goat	1:200	5% NFDM
IL-1β	Santa Cruz	sc-7884	Rabbit	1:200	5% NFDM
IRAK-1 (H-273)	Santa Cruz	sc-7883	Rabbit	1:200	5% NFDM
IRF-3 (FL-425)	Santa Cruz	sc-9082	Rabbit	1:200	5% NFDM
MYD88 (HFL-296)	Santa Cruz	sc-11356	Rabbit	1:200	5% NFDM
p-IRAK-1	Santa Cruz	sc-130197	Rabbit	1:200	5% BSA
TLR2 (H-175)	Santa Cruz	sc-10739	Rabbit	1:200	5% NFDM
TLR3	Enzo	ALX-210-367-R200	Rabbit	1:500	5% NFDM
TLR4 (25)	Santa Cruz	sc-293072	Mouse	1:200	5% NFDM
TRAF6 (H-274)	Santa Cruz	sc-7221	Rabbit	1:200	5% NFDM

(b) Secondary antibodies used for western blots

Antibody	Manufacturer	Catalog #	Dilution
goat anti-rabbit IgG-HRP	Santa Cruz	sc-2301	1:1000
goat anti-mouse IgG-HRP	Santa Cruz	sc-2302	1:1000
donkey anti-goat IgG-HRP	Santa Cruz	sc-2020	1:1000

(c) Primary antibodies used for Immunohistochemistry

Antibody	Manufacturer	Catalog #	Host	Dilution	Diluant	Detergent
IKKi (M-17)	Santa Cruz	sc-5693	Goat	1:50	1X PBS	0.1% Triton
IRF-3 (FL-425)	Santa Cruz	sc-9082	Rabbit	1:50	1X PBS	0.1% SDS

(d) Secondary antibodies used for Immunohistochemistry

Antibody	Manufacturer	Catalog #	Dilution	Diluant
Alexa Flour 488 goat-anti-rabbit	Thermo Scientific	A-11034	1:1000	1X PBS
Alexa Flour 568 donkey-anti-goat	Thermo Scientific	A-11057	1:1000	1X PBS

Table 2.3: Antibodies used for western blots and immunohistochemistry

Antibody names, manufacturers, and dilution information for (a) western blot primary antibodies, (b) western blot secondary antibodies, (c) immunohistochemistry primary antibodies, and (d) immunohistochemistry secondary antibodies.

(a) mRNA and protein changes in PFC

	PFC			
	mRNA		protein- IHC	
	0 hour	24 hour	0 hour	24 hour
Toll-like Receptors and Co-receptors				
Tlr2	–	↑ (1.26)		
Tlr3	–	↑ (2.12)		
Tlr4	–	↑ (1.49)		
Tlr7	↑ (1.15)	–		
Cd14	–	↑ (1.19)		
MyD88-dependent pathway signaling molecules				
Myd88	↑ (1.07)	–		
Irak1	–	–		
pIrak1				
Irak4	↑ (1.19)	–		
Ikkb	–	–		
TRIF-dependent pathway signaling molecules				
Trif	–	↑ (1.23)		
Ikki	↑ (1.17)	–	↑ (1.08)	↑ (1.24)
Irf3	–	↑ (1.56)	–	↑ (1.68)
Molecules involved in both pathways				
Irf7*	↑ (1.18)	–		
Traf3*	–	–		
Traf6	–	–		
Transcriptional outputs of MyD88-dependent pathway				
Il1b	↑ (4.45)	–		
Tnfa	–	–		
Il6	–	↑ (1.58)		
Transcriptional outputs of the TRIF-dependent pathway				
Cxcl10	↑ (2.11)	–		
Ccl5	*	*		
Ifnb	*	*		
Glial Markers				
Cd11b/	–	↑ (1.26)		
Gfap	–	–		
Cd68	↓ (0.89)	–		

(b) mRNA and protein changes in NAc

	NAc			
	mRNA		protein-IHC	
	0 hour	24 hour	0 hour	24 hour
Toll-like Receptors				
Tlr2	–	–		
Tlr3	–	↑ (1.15)		
Tlr4	–	–		
MyD88-dependent pathway signaling molecules				
Myd88	–	–		
TRIF-dependent pathway signaling molecules				
Trif	–	–		
Ikki	–	–	↑ (1.05)	↑ (1.36)
Irf3	–	–	↑ (1.10)	↑ (1.31)
Cytokines and Chemokines				
Il1b	↑ (4.24)	–		
Cxcl10	–	–		

(c) mRNA and protein changes in AMY

	AMY			
	mRNA		protein-IHC	
	0 hour	24 hour	0 hour	24 hour
Toll-like Receptors				
Tlr2	–	–		
Tlr3	–	↓ (0.87)		
Tlr4	–	↓ (0.87)		
MyD88-dependent pathway signaling molecules				
Myd88	–	–		
TRIF-dependent pathway signaling molecules				
Trif	–	–		
Ikki	–	–	–	↓ (0.88)
Irf3	–	–	–	↑ (1.15)
Cyotkines and Chemokines				
Il1b	↑ (3.25)	–		
Cxcl10	–	↓ (0.37)		

Table 2.4: Summary of mRNA and protein changes

Effects of chronic ethanol on mRNA and protein levels in the (a) PFC, (b) the NAc and the (c) AMY were examined 0 and 24 hours after ethanol removal. Effects are shown as increases, decreases, or no change (-); blank cells indicate that mRNA or protein was not measured. Gene names are indicated in the left column, and the numbers in parenthesis indicate fold change compared to control group. PFC, prefrontal cortex; NAc, nucleus accumbens; AMY, amygdala; IHC, immunohistochemistry.

Chapter 3: CNS cell-type localization and LPS response of TLR signaling pathways

Gizelle M. McCarthy B.S., Courtney R. Bridges B.S., Yuri A. Blednov PhD,

R. Adron Harris PhD

3.I. Abstract

Innate immune signaling in the brain has emerged as contributor to many CNS pathologies, including mood disorders, neurodegenerative disorders, neurodevelopmental disorders, and addiction. Toll-like receptors (TLRs), a key component of the innate immune response, are particularly implicated in neuroimmune dysfunction. However, most of our understanding about TLR signaling comes from the peripheral immune response, and it is becoming clear that the CNS immune response is unique. One controversial aspect of neuroimmune signaling is which CNS cell types are involved. Although microglia are the CNS cell-type derived from a myeloid lineage, studies suggest that other glial cell types and even neurons express TLRs, although this idea is controversial. Furthermore, recent work suggests a discrepancy between RNA and protein expression within the CNS. To elucidate the CNS cell-type localization of TLRs and their downstream signaling

molecules, we isolated microglia and astrocytes from the brain of adult mice treated with saline or the TLR ligand lipopolysaccharide (LPS). Glial mRNA expression was compared to a cellular-admixture to determine cell-type enrichment. Enrichment analysis revealed that most of the TLR pathway genes are localized in microglia and increased in microglia following immune challenge. However, expression of *Tlr3* was enriched in astrocytes where it increased in response to LPS. Furthermore, attempts to determine protein cell-type localization revealed that many antibodies are non-specific and that antibody differences are contributing to conflicting localization results. Together these results highlight the cell types that should be looked at when studying TLR signaling gene expression and suggest that non-antibody approaches need to be used to accurately evaluate protein expression.

3.II. INTRODUCTION

Innate immune signaling has been well characterized in the body for decades, but the recent appreciation for its role in the brain has raised several questions. In particular, it has brought to light the similarities and differences between the immune response in the periphery and the central nervous system (CNS). At the center of this discussion, are microglia, the resident immune cells of the brain. However, there is evidence that microglia have unique functions unrelated to immune signaling, and that other CNS cells can also participate in the immune response.

A key component of innate immunity is Toll-like receptors (TLRs), a family of pattern recognition receptors that detect and respond to pathogen and danger signals. TLRs respond to a variety of bacterial and viral pathogens including the bacterial

endotoxin lipopolysaccharide (LPS), which is a ligand for TLR4 (Kawai & Akira 2007). In response to LPS, TLR4 with its co-receptor cluster of differentiation 14 (CD14) can signal through two distinct pathways, the Myeloid differentiation primary response protein 88 (MyD88)-dependent pathway and the TIR-domain containing adaptor protein inducing IFNβ (TRIF)-dependent pathway (Takeda & Akira 2004) (**Fig. 3.1**). The MyD88-dependent pathway signals through Interleukin 1 receptor associated kinases 1 and 4 (IRAK1 and IRAK4) and TNF receptor associated factor 6 (TRAF6) leading to activation of inhibitors of nuclear factor Kappa B Kinases (IKKs) (Kawai & Akira 2007). Activation of IKKs causes activation of NF-κB and the production of pro-inflammatory cytokines (e.g. TNF, IL-1β, IL-6). In contrast, the TRIF-dependent pathway utilizes the adaptor protein TRIF and signals through TRAF3, TBK1, and IKKε leading to phosphorylation and activation of interferon regulatory factor 3 (IRF3) (Takeda & Akira 2005). Activated IRF3 translocates to the nucleus where it leads to the transcription of type I interferons and interferon inducible genes (e.g. IFN-β, CCL5/RANTES, CXCL10/IP-10).

TLR signaling has been implicated in several CNS conditions, including ischemia, neurodegeneration, depression, and addiction (García Bueno et al. 2016; Gąsiorowski et al. 2017; Gambuzza et al. 2014; Gesuete et al. 2014; Crews et al. 2017; Hanke & Kielian 2011). However, the cell-type localization of TLR signaling within the CNS remains controversial and impairs our understanding and ability to develop treatments based on these signaling pathways. TLR signaling was originally

characterized in peripheral immune cells, thus, it was believed that CNS expression of TLRs would be limited to microglia, the immune cells of the brain. Several studies support microglial expression of TLRs, and many reaffirm the idea that expression is completely or mostly microglial (Bsibsi et al. 2002; Lehnardt et al. 2002; Olson & S. D. Miller 2004; Hanke & Kielian 2011). However, recent studies suggest that TLRs are also expressed and functionally important in other glial cells like astrocytes and oligodendrocytes (Kielian 2006; Gorina et al. 2010; Borysiewicz et al. 2013; Park et al. 2005; Blanco et al. 2005; Marinelli et al. 2015; Lehnardt et al. 2002; Bsibsi et al. 2002) or even non-glial CNS cells like neurons (Xing-Jun Liu et al. 2016; Aurelian et al. 2016; Peltier et al. 2010; Préhaud et al. 2005; Qi et al. 2011; Crews et al. 2013). These results are complicated by differences in methodology across studies, including differences in: protein or mRNA; *in vivo*, primary cells or in established cell lines; species; and techniques. Interestingly, there seems to be disagreement between mRNA and protein expression for the same molecule, which raises many questions. For example, a brain RNA expression database shows Tlr4 as highly microglial (Y. Zhang et al. 2014), while the human protein atlas (proteinatlas.org) shows it only detected in neurons (Uhlén et al. 2015). Several other TLR signaling molecules (MyD88, IRAK1, TRIF, IRF3) also show highest mRNA expression in microglia, but highest protein expression in neurons.

Although many studies have reported the localization of TLRs in the CNS, few have evaluated the expression of the downstream signaling molecules and pathway outputs that are responsible for functional changes. It is also remains unclear how immune activation might change cell-type expression of TLR signaling *in vivo*, as most

studies have evaluated the response to TLR agonists using cultured cells (Lawrimore & Crews 2017; Marinelli et al. 2015; Rosenberger et al. 2014; Hanke & Kielian 2011; June et al. 2015). Recent studies suggest that established cell-lines and even primary cultured glial cells don't accurately reflect the expression profile *in vivo* (Butovsky et al. 2013).

Although this discrepancy may seem esoteric, it is a major hindrance to the study of neuroimmune signaling. In our lab alone, we have had several problematic studies because it was unclear which cell type to use for a conditional knockout or viral vector, or a gene was knocked out in microglia but couldn't be verified on the protein level because of neuronal expression. These uncertainties not only result in wasted time and money, but delay the discovery of important results. Given the key role of TLR signaling in CNS pathologies, and the desire to manipulate and understand these pathways in the brain, it is imperative that cell-type localization of these molecules is determined and agreed upon.

Based on the disagreement in the field and preliminary results that suggested TLR-signaling mRNAs are localized in microglia while protein is localized in neurons, we sought to investigate TLR signaling localization using glial cells isolated from adult mouse brain. The goals of this study were to identify the cell-type enrichment of TLR pathway mRNAs and proteins with and without immune activation (LPS treatment) and to determine which cells exhibit expression changes following activation. There is literature supporting the idea of that cell-type protein expression can change after LPS (Béchade et al. 2014), so we hypothesized that key mRNAs will be abundant in microglia so to allow rapid translation into protein in response to immune activation. Our results

revealed that mRNA was primarily microglial, although there were some differences in expression profiles, and that LPS increased mRNA expression in microglia. In contrast, our protein results were inconclusive due to non-specific antibodies and conflicting results across antibodies for the same protein. Based on our results, we conclude that much of the disagreement in the field is due to antibody failures, and that better antibodies or alternative methods need to be developed to conclusively determine protein localization in brain cells.

3.III. Materials and Methods

3.III.a. Ethics Statement

All procedures were approved by the University of Texas at Austin Institutional Animal Care and Use Committee (animal protocol number AUP-2013-00061) and adhered to the NIH Guidelines. The University of Texas at Austin animal facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

3.III.b. Animals and LPS administration

Studies were conducted in adult (6-8 weeks old) C57Bl/6J male mice (Jackson Laboratories, Bar Harbor, ME). Mice were individually housed and allowed to acclimate to upright bottles one week before the start of the experiment. The experimental rooms were maintained at an ambient temperature of 21±1°C, 40-60% humidity, and a regular light/dark schedule (7 AM-7 PM). Food and water were available *ad libitum*. The mice

were divided into 3 groups, each containing 7 LPS treated mice and 5 saline treated mice (additional mice were put in the LPS group in case of death before 24 hours) (**Fig. 3.2**). The mice were weighed and had water intake measured for 2 days prior to injection and then were injected with either LPS (2.0 mg/kg) or saline. Mice were weighed and water intake was measured 24-hours post-injection and the mice were sacrificed. Weight and water consumption data is provided in **Figure 3.3**.

3.III.c. Knockout Animals

Knockout (null mutant) mice for TLR2, TLR4, and MyD88 are described in (Blednov et al. 2017). Briefly, the TLR2 knockout mouse was B6.129S1-*Tlr2*^{tm1Dgen}/J (Jackson Laboratories, Bar Harbor, ME) which has a neomycin cassette inserted in the gene making it non-functional (Werts et al. 2001). The TLR4 knockout mouse was B6.B10Scn-Tlr4$^{lps\text{-}del}$/JthJ (Jackson Laboratories), which has the locus containing the *Tlr4* gene deleted (Poltorak et al. 1998). The MyD88 knockout mouse was B6.129P2(SJL)-*MyD88*$^{tm1.1Defr}$/J (Jackson Laboratories), and is a cross of Myd88^{tm1Defr} mice (*loxP* sites flanking exon 3 of *Myd88*) with Tg(Zp3-cre)93Knw mice (Hou et al. 2008). RT-qPCR was used to determine the transcript expression in the knockout mice (**Fig. 3.4**). The TLR4 knockout mouse showed no transcript expression, consistent with previous studies (Poltorak et al. 1998). The MyD88 knockout mouse showed decreased expression of *MyD88*, likely due the fact that only exon 3 is removed and the primers are not on exon 3. The TLR2 knockout mouse showed increased expression of *Tlr2*, which is consistent

with a larger transcript being produced due to the neomycin cassette (Wooten et al. 2002).

3.III.d. Tissue Harvest and microglial isolation

5 mice per group were perfused with ice-cold saline and the brain was removed (each group was performed on a different day). The dissected tissue was pooled by treatment within group (ie. all of group 1 saline samples were combined, see **Fig. 3.2**). The reason for pooling samples was to get enough microglia to get enough cells for both qPCR and western blots. Approximately 1% of the minced tissue was taken as a total homogenate (TH) sample that includes all cell types. The total homogenate was further divided into 10% for RNA and 90% for protein and centrifuged at 1000 x g for 10 minutes at 4°C. The supernatant was removed and the cells were flash frozen in liquid nitrogen. The remaining sample was used for microglial isolation as described by Nikodemova et al. 2015. Briefly, tissue suspension was enzymatically dissociated using the Neural Tissue Dissociation Kit- Papain (Miltenyi Biotec, Germany) in conjunction with Pasteur pipette manual dissociation. Dissociated tissue was passed through a 70 μM strainer (Miltenyi Biotec), centrifuged at 300 x g, then resuspended in 30% percoll (Sigma-Aldrich, St. Louis, MO). The percoll-cell suspension was centrifuged at 700 x g for 15 minutes at room temperature with the myelin fraction removed from the top fraction. Cells were washed and then incubated with CD11b MicroBeads (Miltenyi Biotec) and eluted using MS columns to collect CD11b+ cells. Cells were again divided (10% for RNA and 90% for protein) and CD11b+ cell pellets were collected by

centrifugation at 300 x g for 10 minutes at 4°C and then flash frozen. The CD11b- fraction was also spun down and the pellet was resuspended in astrocyte-binding ACSA2 MicoBeads (Miltenyi Biotec). The ACSA2+ fraction was collected as the CD11b+ fraction was, and the remaining negative fraction (CD11b/ACSA2-) and the astrocyte fraction (ACSA2+) were divided (10% for RNA, 90% for protein), spun down and pellets were flash frozen.

3.III.e. RNA Isolation and qPCR

RNA was isolated from all four fractions (TH, CD11b+, ACSA2+, CD11b/ACSA2-) using the MagMax-96 Total RNA Isolation Kit (Thermo Fisher Scientific Inc., Rockford, IL). The RNA yield was quantified on a NanoDrop 1000 spectrophotometer and assessed for quality on an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA). RNA was reverse transcribed into cDNA using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc.). cDNA was tested for genomic DNA contamination and showed at least a 10 Cq difference between the +RT (reverse transcription) and –RT samples (Bustin et al. 2009). Applied Biosystems TaqMan® Gene Expression Assay (Thermo Fisher Scientific Inc.) primers were used, and specific assay IDs are shown in **Table 3.1**. RT-qPCR reactions were performed using SsoAdvanced™ Universal Probes Supermix (BioRad, Hercules, CA) in 10-µL reactions containing 250 pg of cDNA. All reactions were performed in technical triplicates for each biological replicate and included a negative no-

template control. Samples were normalized to 18s and relative expression was determined using the CFX software (BioRad).

3.III.f. Protein isolation and western blots

Cells or Tissue were homogenized in lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1% Triton-X-100, 1% sodium deoxycholic acid, 0.1% SDS, 1X Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific Inc.), rocked for 30 minutes at 4°C, centrifuged for 10 minutes at 10,000 x g, aliquoted, and frozen at -80°C. HEK-293 cells were kindly provided by Dr. Mihic's laboratory. Cells were washed with cold PBS, scraped and washed with lysis buffer, and processed as described above. Protein concentrations were determined using the DC Protein Assay (Bio-Rad). Cell lysates (20 µg for fractions, 40 ug for antibody tests) were boiled for 5 minutes, run on 4-15% Mini-Protean TGX Precast Gels (Bio-Rad), and transferred to PVDF membranes using semi-dry transfer. All fraction blots contained a control sample (mouse whole brain lysate) for normalizing across blots. Membranes were blocked with 5% dried milk in TBST (Tris-buffered saline with 0.5% Tween-20) and incubated overnight at 4°C with primary antibody (**Table 3.2**). Membranes were washed with TBST and incubated with HRP-conjugated secondary antibodies in 5% dried milk in TBST (**Table 3.2**). Bands were visualized using ECL (Pierce) and imaged on film and using G:BOX Chemi XX6 (Syngene, Cambridge, UK). Attempts were made to identify a loading control that was equal across all cell types, but every loading control examined showed differences in expression across fractions.

3.III.g. Combined Fluorescent in situ hybridization and immunohistochemistry

Protocol adapted from Exiqon miRCURY microRNA ISH Optimization Kit. Mice were transcardially perfused with 4% paraformaldehyde (PFA), and the brains were post fixed overnight in 4% PFA at 4°C and transferred to 30% Sucrose overnight at 4°C. Brains were fresh frozen and coronally sectioned on a cryostat (20uM). Free-floating sections were post-fixed in 10% NBF overnight at room temperature. After three 1x PBS washes (3 minutes per wash), slices were hybridized with a double DIG-labeled custom Locked Nuclei Acid (LNA) probe (Exiqon) for 1 hour at appropriate hybridization temperature (**Table 3.3**). Following hybridization, slices were washed in 5x SSC, 1x SSC (2 times), and 0.2x SSC (2 times) at same temperature as hybridization for 5 minutes per wash. After a final 0.2x SSC wash at room temperature for 5 minutes, slices were blocked with blocking solution (1x PBS, 0.1% Tween-20, 2% donkey serum, and 1% BSA) at room temperature for 15 minutes. Slices were then incubated in anti-DIG antibody (for mRNA probe) and appropriate primary antibody for protein of choice (**Table 3.3**) overnight at 4°C. All antibodies were diluted in antibody solution (1x PBS, 0.05% Tween-20, 1% donkey serum, and 1% BSA). After 3 1x PBS-T (0.1%) washes (3 minutes per wash), appropriate secondary antibodies were applied to the slices and allowed to incubate at room temperature for 1.5 hours. After three final 1x PBS washes (10 minutes per wash), Finally, slices were mounted on charged slides and counterstained with DAPI (Fluoromount-G, Southern Biotech). Slides were visualized on a Zeiss Axiovert 200M Fluorescent Microscope and analysis was completed on Photoshop CC5 (Adobe) and ImageJ. Probe and antibody information is found in **Table 3.3**.

3.III.h. Immunohistochemistry

Brains were prepared as stated above and free floating sections were placed into PBS. Sections were permeabilized in detergent (.1% Triton-X-100) and blocked in 10% goat or donkey serum for 1 hour at room temperature. Antibody treatment and mounting was performed as described above. Antibody information is in **Table 3.3**.

3.III.i. Statistical Methods

RT-qPCR data was analyzed with a 2-way analysis of variance (ANOVA) and Tukey's multiple comparisons test.

3.IV. RESULTS

3.IV.a. Fraction mRNA cell-type enrichment

Four fractions were collected from the saline and 24-hour LPS treated samples: TH (total homogenate), CD11b+ (microglial fraction), ACSA2+ (astrocyte fraction), and CD/AC- fraction (cells remaining after isolation of microglia and astrocytes, referred to as the negative fraction). RT-qPCR was performed using cell-type markers to determine the cell-type enrichment for each of these fractions (**Fig. 3.5**). *Cd11b*/*Itgam* was used as a marker for microglia and expression was found to be highly expressed in the CD11b+ fraction, lowly expressed in the TH, and absent in the ACSA2+ and CD/AC- fractions (**Fig. 3.5A**). *Glast*/*Slc1a3* was used as an astrocyte marker and was found to be lowly expressed in the TH and highly expressed in the ACSA2+ fractions under saline conditions (**Fig. 3.5B**). *Neun* was used as a neuronal marker and was expressed at high

levels in the TH and low levels in the CD/AC- fraction, expression was absent in the CD11b+ and ACSA2+ fractions (**Fig. 3.5C**). The reason for the lack of neuronal markers in the negative fraction is that adult neurons don't survive the isolation procedure well, therefore, the TH taken before isolation contains the most neurons. *Tek* was used as a marker for endothelial cells and was highly expressed in the CD/AC- fraction and lowly expressed in the TH, CD11b+ fraction and ACSA2+ fraction (**Fig. 3.5D**). *Tek* expression decreased significantly in the CD/AC- fraction following LPS. *Cd68* was used as a marker of activated microglia and was highly expressed in the CD11b+ fraction and increased following LPS treatment (**Fig. 3.5F**).

3.IV.b. Tlr mRNA cell-type localization and LPS response

qPCR was used to evaluate the expression of the most widely studied Tlrs, *Tlr2, Tlr3, Tlr4,* and the TLR4 co-receptor, *Cd14*. Under basal conditions, expression of *Tlr2*, *Tlr4* and the co-receptor Cd14 was primarily localized to microglia, as evidenced by the high SAL-CD11b+ expression compared to SAL-TH expression (**Fig. 3.6**). In response to LPS, *Tlr2* and *Cd14* expression increased in microglia, 4-fold and 2.6-fold respectively. Alternatively, *Tlr4* expression decreased by approximately 50% in microglia following LPS (**Fig. 3.6C**). In contrast to *Tlr2*, *Tlr4*, and *Cd14*, *Tlr3* was expressed in all fractions, with highest expression in astrocytes. In response to LPS, *Tlr3* expression increased in astrocytes, but not in any of the other fractions (**Fig. 36B**). No *Tlr* expression changes were detected in the total homogenate.

3.IV.c. MyD88-dependent pathway mRNA localization and LPS response

To determine localization and LPS response, mRNA expression of components of the MyD88-dependent pathway (*Myd88*, *Irak1*, *Irak4*, *Traf6*, and *Ikkb*), as well as cytokines produced in response to MyD88-pathway activation (*Il1b*, *Il6*, *Tnf*) was measured (**Fig. 3.7**). While all MyD88-dependent pathway genes were expressed highest in microglia under basal conditions, the expression patterns were variable. *Myd88* and *Irak4* displayed low basal expression in other fractions, while *Irak1*, *Traf6*, and *Ikkb* were expressed at greater than 50% of the expression level of microglia, suggesting expression in astrocytes and endothelial cells as well. In contrast, the cytokines were almost exclusively expressed in microglia (**Fig. 3.7F-H**). In response to LPS, *Myd88* expression increased in microglia while *Irak4* decreased (**Fig. 3.7A, C**). Interestingly *Traf6* increased in astrocytes and the CD/AC- fraction while *Irak1* trended towards an increase in astrocytes ($p=.02$ in t-test but not significant when corrected for multiple comparisons). Both *Il1b* and *Tnf* increased in microglia following LPS administration, however, *Tnf* increasing almost 14-fold. In contrast, *Il6* expression did not increase in microglia, but trended towards an increase in astrocytes and the CD/AC- fraction ($p = .04$ astrocytes and $p= .03$ CD/AC-, uncorrected t-test) (**Fig. 3.7G**).

3.IV.d. TRIF-dependent pathway mRNA localization and LPS response

Expression of TRIF-dependent pathway components (*Trif, Traf3, Ikki*, *Irf3*) and outputs (*Ifnb, Ccl5, Cxcl10*) were measured under basal conditions and in response to LPS to allow comparison with the MyD88-dependent pathway (**Fig. 3.8**). *Trif* and *Irf3* had similar basal expression profiles with highest expression in microglia, but *Irf3* was enriched in the astrocyte fraction and in the negative fraction as well and *Trif* showed modest expression in all fractions. *Traf3* and *Ikki* were expressed relatively evenly across the fractions under basal conditions, although *Ikki* trended towards highest expression in astrocytes ($p<.0001$ using 1-way ANOVA for saline group). Under basal conditions, *Ifnb, Ccl5, and Cxcl10* are virtually undetectable in all fractions except for some *Ccl5* expression in microglia and some *Cxcl10* expression in microglia and astrocytes. In response to LPS, *Trif* and *Irf3* expression decreased in microglia, while *Traf3* expression decreased in the TH. In contrast, *Ikki* showed 23.5-fold increase in expression following LPS. Like *Ikki*, *Ifnb* and *Ccl5* increased in microglia, while *Cxcl10* trended towards an increase in microglia and astrocytes ($p=.052$).

3.IV.e. Antibody validation in knockout tissue and HEK-293 cells

Knockout mice for TLR2, TLR4, and MyD88 were available in the lab, so they were used to test the specificity of the antibodies for those proteins (**Fig. 3.9**). In addition, HEK-293 cell lysates were used for validation because these cells should not express TLR2, TLR3, TLR4, or IL-1β (www.proteinatlas.org) (Uhlén et al. 2015). Testing with the TLR2 antibody revealed expression in wild-type brain tissue, HEK-293 cells, and

TLR2 knockout tissue, suggesting non-specific binding (**Fig. 3.9A**). The TLR3 antibody showed strong expression (although at a lower molecular weight than expected) in the WT brain tissue, and no expression in the 293 cells (**Fig. 3.9B**). Two TLR4 antibodies were tested and both produced signal in the 293 lysates and in the TLR4 knockout tissue (**Fig. 3.9C-D**). Furthermore, the TLR4 (76B357.1) antibody appeared to run at a lower molecular weight than anticipated, although there were multiple bands that appeared at different molecular weights in each lysate (**Fig. 3.9C**). The IL-1β antibody produced a strong signal in the 293 lysates, suggesting it is also non-specific (**Fig. 3.9E**). Five MyD88 antibodies from 2 different companies were tested in MyD88 knockout tissue (**Fig. 3.9F-J**). All 5 antibodies produced a signal in the knockout tissue, and sc-74532 appeared at the incorrect molecular weight, indicating that none of these antibodies were specific. These tests suggested that most the antibodies that we tested were non-specific, and made us skeptical of the ones we could not test in knockout tissue. Responses from the antibody vendors indicated that antibodies were never tested against negative controls, only against blocking peptides.

3.IV.f. Fraction protein localization in western blots

Because antibody specificity could not be verified, full replicates of western blots were not performed and thus, not quantified. However, sample western blot images for each antibody are shown in **Fig. 3.10** to demonstrate the variety of expression profiles and how different antibodies to the same protein produce different results (**Fig. 3.10**). First, as with qPCR, cell-type marker expression was evaluated in the lysates using

antibodies for NEUN (neuronal marker), GFAP (astrocyte marker), and IBA1(microglial marker) (**Fig. 3.10A**). Differences in markers between qPCR and western blots were due to antibody availability and efficacy. Consistent with the qPCR data, NEUN was present in high amounts in the control sample and the total homogenate sample, but not in other fractions. GFAP was expressed in the control sample and the TH, but expressed highest in the astrocyte fraction, like the qPCR results. IBA1 was expressed very strongly in microglia and could be seen in the control and TH after a much longer exposure that left the microglial expression overexposed. These findings are consistent with the qPCR data which shows that expression of microglial markers is over 50x higher in the microglial fraction than the TH.

Despite the determination that many of the antibodies were non-specific, localization of TLR protein and IL-1β was investigated to see if these results mirrored some of the confusing data in the literature suggesting non-microglial localization (**Fig. 3.10B**). Even though *Tlr2* mRNA expression was predominantly microglial, TLR2 protein was detected in every fraction except microglia. TLR3, which was found to be microglial and astrocytic on the mRNA level, was found exclusively in the TH on the protein level, suggesting neuronal localization. TLR4 and IL1β were highly expressed in microglia on the mRNA level, but were expressed in all fractions on the protein level. Furthermore, IL-1β expression was lowest in microglia. These data suggest that studies detecting neuronal localization of TLRs, despite microglial mRNA, may be due to non-specific antibodies.

Because we had so many antibodies that claimed to detect MyD88, this presented an opportunity to compare localization of the same protein using different antibodies (**Fig. 3.10C**). MyD88 (sc-11356) was expressed only in the TH, suggesting neuronal expression. In contrast, ab2064 and ab2068 were expressed in all fractions, although highest in microglia and in the negative fraction. MyD88 (sc-8197) gave such strange results with vastly different molecular weight bands across the fractions, that is was not included. MyD88 (sc-74532) was not used because tests revealed that the signal was at the wrong molecular weight (**Fig. 3.9H**). These results were particularly concerning because every antibody tested in the knockout was non-specific and different antibodies produced different results.

The rest of the MyD88-pathway produced equally confusing results. Like TLR2, IRAK1 protein was expressed in every fraction except microglia. TLR4 showed highest expression in the TH, but faint expression in other fractions at a slightly lower molecular weight. For TRAF6 we had two antibodies from the same company, sc-8409 (monoclonal mouse) and sc-7221 (rabbit polyclonal). Both antibodies produced several bands (**Fig. 3.10E**), making it difficult to determine what signal was real. TRAF6 should run at 60 kD, which corresponds to the middle band on the sc-8409 blot and top and on the sc-7221 blot. Based on these bands, expression appears to be highest in the TH and the astrocyte fraction. IKKβ was primarily localized to the TH (**Fig. 3.10F**), which is consistent with the neuronal localization seen in immunohistochemistry data from our lab (Truitt et al. 2016), but inconsistent with the qPCR data.

Protein expression evaluation was limited for the TRIF-dependent pathway (due to antibody challenges) and expression of IRF3 and IKKε was determined (**Fig. 3.10G-H**). IRF3 was expressed in all fractions, but highest expression was in the negative fraction. IKKε was evaluated using 2 antibodies, sc-5693 (goat polyclonal) and sc-376114 (mouse monoclonal). IKKε sc-5693 is a very weak antibody, but detected some protein in the control sample and total homogenate (the bands in the astrocyte fraction are suspected to be bleed through). IKKε (sc-376114) is supposed to be expressed at 80 kD, which corresponds to the top band, however, the multiple bands raise concerns.

3.IV.g. Protein and RNA expression in tissue sections

Several of the proteins evaluated with western blot have also been investigated in brain tissue using immunohistochemistry with the same or different antibodies. An example of these are shown in **Figure 3.11**. Immunohistochemistry reveals highly neuronal expression in tissue for MyD88 (sc-8197), IRAK1 (sc-7883), and TRAF6 (sc-7221). These results are relatively consistent across TLR-pathway antibodies that have been tested in our lab (high neuronal staining). Interestingly, attempts to look at *Irf3* mRNA expression also suggested neuronal localization (**Fig. 3.12**). Because we knew that *Irf3* mRNA should be in microglia, we tested a microglial marker, *Tmem119* (Bennett et al. 2016), using the same protocol. *Tmem119* also failed to express in microglia (**Fig 3.12C-D**), suggesting that there may be a permeability issue when targeting glial cells in tissue, resulting in high background staining in neurons. It is worth

noting that *Irf3*, which is more heterogeneous across cell types showed a much stronger neuronal signal than *Tmem119*, which should only be in microglia. This suggested to us that the small amount of *Irf3* localized in neurons was all we could detect, while the detected neuronal *Tmem119* was just background due to increased probe concentrations.

3.V. DISCUSSION

TLR signaling is a key component of the innate immune response and it contributes to many brain disorders, including alcohol use disorders. However, the cell-type specific response to immune stimuli in the CNS remain unclear. Identification of the cell-type localization of TLR signaling and immune response within the brain is necessary to elucidate the functional implications of perturbed signaling and to design future studies with *in vivo* manipulations. To address this, we used isolated glial cells from adult mice that been administered either saline or LPS. Using four distinct cell-fractions, we evaluated the mRNA expression of TLRs, their downstream signaling molecules, and the transcriptional outputs of their signaling (**Table 3.4, Fig. 3.13**). In addition, we tried to profile the protein expression of TLR signaling molecules. Unfortunately, we were not able to draw any conclusions about the protein localization, but we have identified reasons why there may be disagreement in the field.

Although expression of mRNA from the TLR signaling pathway was primarily microglial as expected, there were extremely variable expression profiles within the pathways. This is consistent with gene expression data from adolescent (P17) mice in the

RNA-Seq transcriptome database (Y. Zhang et al. 2014). While expression of *Tlr2, Tlr4, and Cd14* were highly microglial, expression of *Tlr3* was highest in astrocytes, where expression increased in response to LPS. These findings are consistent with several studies that have shown *Tlr3* to be expressed and functional in astrocytes (Park et al. 2005; Scumpia et al. 2005; Borysiewicz et al. 2013; Jack et al. 2005) as well as a study that shows *in vitro* LPS increases *Tlr3* expression in primary astrocyte cultures while decreasing *Tlr3* expression in primary microglial cultures (Marinelli et al. 2015). TLR3 signals through the TRIF-dependent pathway, however, the components of the TRIF-dependent pathway showed varied expression and LPS responses. This raises the question of how signaling molecules within a pathway could be expressed in different cell types. Although *Trif* and *Irf3* expression is highest in microglia, there is still significant expression in astrocytes, and *Ikki* expression trends towards being mostly astrocytic under basal conditions. Therefore, it is possible that signaling is occurring in both cell-types and that the mRNA expression of the receptor and its signaling molecules are not 1:1 within the cell. Furthermore, microglial *Trif* and *Irf3* expression decrease following LPS, while *Ikki* expression increases, suggesting they are independently regulated. This is supported by the involvement of *Ikki* in other LPS-responsive pathways (e.g. JAK/STAT signaling), that could have different cell-type specificity.

It is surprising that the expression of *Ifnb* and *Ccl5* is exclusively microglial. There is a trend towards increased expression of *Cxcl10* in both astrocytes and microglia after LPS, suggesting that the TRIF-dependent pathway is being activated and inducing downstream signaling in both cell types. This raises the question of how expression of

Cxcl10 is increased without *Ifnb* there to induce it. It is possible that interferon-inducible genes are produced in response to IFNβ in microglia, but changed in a different manner in astrocytes which lack a macrophage lineage. Astrocytes produce interferon in a TLR3 and TLR4 dependent manner *in vitro* (Reinert et al. 2012; Pascual-Lucas et al. 2014), but it is possible that astrocytes respond differently *in vivo*. It is also plausible that the inflammatory response is temporally mediated within cell-type, and that increased expression of interferons would be detected in astrocytes if evaluated earlier or later. It is noteworthy that TLR4 also signals through the TRIF-dependent pathway and is highly microglial, so perhaps TLR3 signaling is predominant in astrocytes, while TLR4-induced TRIF dependent signaling dominates in microglia leading to the increased *Ifnb, Ccl5, and Cxcl10* seen in the CD11b+ fraction.

Expression of components of the MyD88 pathway were highest expressed in microglia, consistent with expression of *Tlr2* and *Tlr4*, which signal through the MyD88-dependent pathway. However, although *Tlr2* and *Tlr4* expression was highly enriched in microglia, some components of the MyD88-dependent pathway (*Irak1, Traf6, Ikkb)* were more evenly distributed across the fractions and *Traf6* expression increased in the astrocyte fraction following LPS. The different expression profiles could be because *Traf6* can also be activated via TRIF in response to TLR3 or TLR4, and is involved in other pathways like TGF-β signaling. *Ikkb* is also involved in every pathway that signals to NF-κB, not just TLR pathways. Consistent with the notion that MyD88-dependent signaling is mostly occurring in microglia, *Il1b, Il6, and Tnf* expression were primarily

microglial. However, there is a trend towards an increase in *Il6* seen in astrocytes and the CD/AC- negative fraction in response to LPS. There is evidence that *Il6* is also activated in response to LPS and TRIF-dependent signaling in cultured astrocytes(Marinelli et al. 2015; Jack et al. 2005), so it is possible that TRIF-dependent increases in *Il6* expression occur in astrocytes *in vivo*.

It is worth noting that although several robust changes were observed in response to LPS within the cell fractions, none were observed in the total homogenate, which is the typical preparation for evaluation of gene expression. This highlights the importance of looking at discrete cell types when evaluating immune changes in the brain, particularly because expression could be decreasing in one cell type while increasing in another (as seen with *Tlr3)*. A caveat to this is that even whole brain samples had to be pooled to get enough RNA for RT-qPCR. Because of this, any brain-region specific changes are missed and the statistical power is reduced. Furthermore, the primers used for RT-qPCR are designed to target a single exon-exon junction, so exon level expression and splice variants may be missed.

Although expression of mRNA and protein is not always 1:1, we were unable to find any examples in the literature of all the mRNA residing in one cell-type while all the protein is in another cell-type. Because this is what our preliminary data suggested, we sought to test our hypothesis that many copies of mRNA were found inside microglia to ensure rapid translation in response to danger signals, although this hypothesis did not address why protein was found in neurons. After this study, we are just as unclear, if not

more, about the protein localization of TLR signaling molecules. However, we do have some thoughts as to what is causing this confusion.

The western blots we performed show incredibly variable expression profiles, but the most concerning result is that several TLR signaling proteins do not appear to be expressed in microglia (TLR2, TLR3, MyD88 sc-11356, Irak1, IKKβ), even though all of them show microglial mRNA expression and most show highest expression in microglia. However, after some quality control steps, we are unable to trust any of the protein results. For the antibodies we could test, all but one showed expression in the negative control. For the antibodies we were unable to test on null mutant tissue, we erred on the side of caution and assumed they are also non-specific. Furthermore, different antibodies to the same protein gave very different expression profiles (**Fig. 3.9C**), reaffirming that the antibodies cannot be trusted. We suspect that antibody specificity is one of the major reasons for disagreement in the field. Even though other researchers have told us that TLR antibodies are notoriously non-specific among, they continue to be used in publications and these results continue to be cited as accurate. Even resources like the human protein atlas use antibodies to determine cell type localization (Uhlén et al. 2015). For example, the data for MyD88 in the human protein atlas suggests that protein is highly neuronal, but RNA expression is mostly glial. Interestingly, the antibody they use is sc-11356, which I found to be non-specific (**Fig. 3.8F**). They do provide information about the antibody validation, but they are basing the validation on comparison of staining in one tissue type (colon) to the literature.

It isn't surprising that antibodies are non-specific given that most manufacturers only validate them in transfected cell lines or with blocking peptide. Some companies, when asked, could not even suggest a negative control and claimed it would be too difficult to test all antibodies on knockout tissue. Unless this practice changes, every lab needs to test the antibody in their hands with positive and negative controls to be confident their results are accurate. Due to the difficulty of testing several antibodies for each protein, other approaches may be better suited for looking at several proteins at once. Proteomic approaches in glial cells have revealed protein changes that more closely match what is expected (Bell-Temin et al. 2013). Alternatively, construction of transgenic mice with GFP-tagged expression of TLR genes may be useful to show the CNS cell-type localization.

In addition to our western blots, our immunohistochemistry and *in situ* results suggest that glial cells are less likely to be permeable to probes or antibodies. Therefore, more stringent permeabilization steps may be needed to detect intracellular molecules in glia. Although we attempted different permeabilization steps using the Exiqon protocol, we continued to see neuronal localization. However, using the RNAscope® (Advanced Cell Diagnostics, Inc., Newark, CA) protocol, we could identify *Tmem119* in microglia. In addition, we can see microglial staining with immunohistochemistry using the IBA1 antibody (Wako), but several other microglial antibodies don't work well. This further suggests that some antibodies are better able to get through the cell membrane, and this idea has been acknowledged in other studies (Melvin & Sutherland 2010).

In conclusion, this study confirms and expands on mRNA cell-type localization of TLR signaling molecules and evaluates cell-type specific increases following LPS administration. This study was unable to reliably determine the protein localization of TLR signaling molecules, and we suggest this is due to non-specific antibodies and problems with permeabilization. We suggest that future studies evaluating cell-type expression take these results into account and that perhaps other non-antibody approaches be used to determine the protein localization of this important pathway in the CNS.

3.VI. Acknowledgements

The authors thank Olga Ponomareva, Jillian Benavidez, Mendy Black, and Adriana DaCosta for their technical assistance.

3.VII. FIGURES

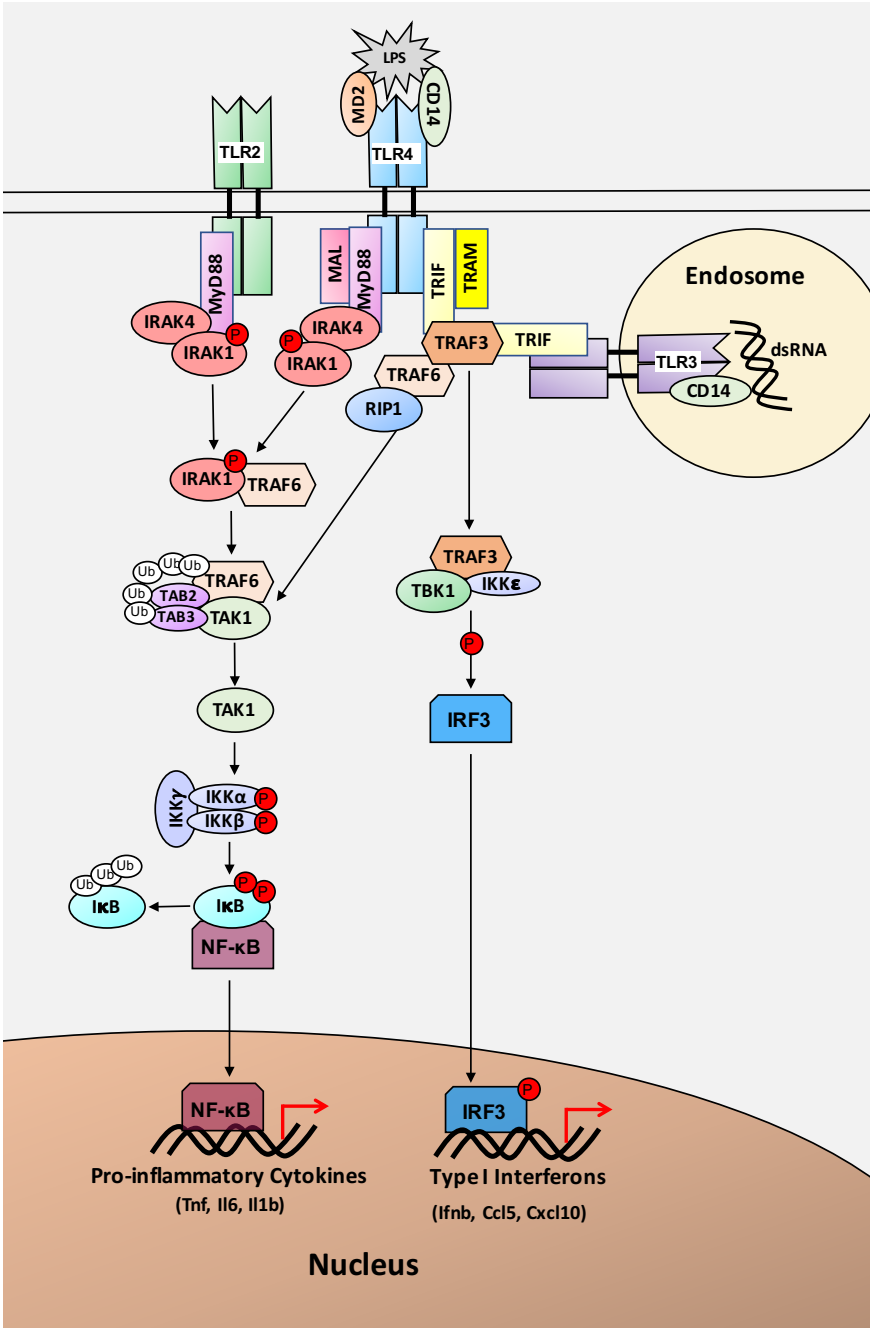


Figure 3.1: TLR-signaling pathways

Figure 3.1: Continued

Lipopolysaccharide (LPS) is recognized by TLR4 and its co-receptors MD2 and CD14. TLR4 signals through two different pathways, the MyD88-dependent pathway and the TRIF-dependent pathway. The MyD88-dependent pathway utilizes the adapter protein MyD88, which recruits IRAK4, IRAK1, and TRAF6. Phosphorylation of IRAK1 and ubiquitination of TRAF6 leads to activation of IKKs and NF-κB. Activated NF-κB translocates to the nucleus where it promotes transcription of pro-inflammatory cytokines. TLR2 also signals through the MyD88-dependent pathway. The TRIF-dependent pathway, utilized by TLR3 and TLR4, signals through the adapter protein TRIF. TRIF recruits TRAF6 and TRAF3. Signaling through TRAF6 leads to NF-κB activation, while signaling through TRAF3 utilizes IKKε to activate IRF3. Activated IRF3 translocates to the nucleus where it leads to transcription of Type I interferons and interferon inducible genes.

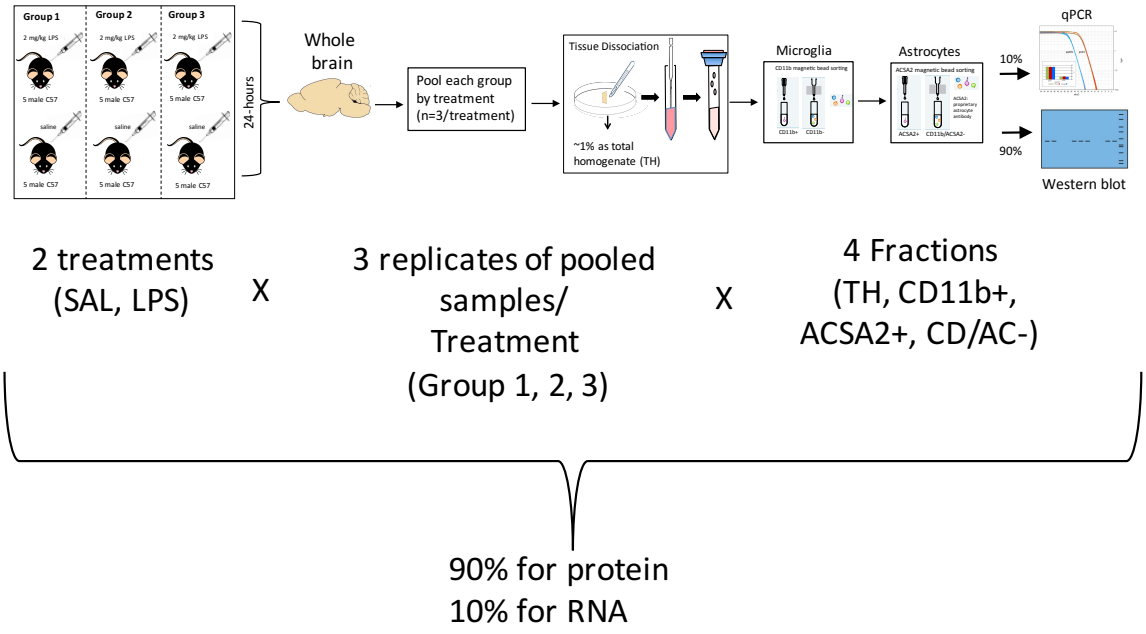


Figure 3.2: Schematic of study methods

The mice were divided into three subgroups, each containing 5 mice per treatment. Mice were injected with either saline or 2 mg/kg LPS and sacrificed 24-hours later. The whole brain was removed and tissue was pooled within each group by treatment yielding 3 biological replicates per treatment. 1% of minced tissue was taken as total homogenate and the remaining tissue was used to isolate microglia (Cd11b+) and astrocytes (Acsa2+). The remaining cells (Cd11b/Acsa2-) were also collected. 10% of each sample was used for RNA isolation and RT-qPCR and 90% was used for protein isolation and western blots.

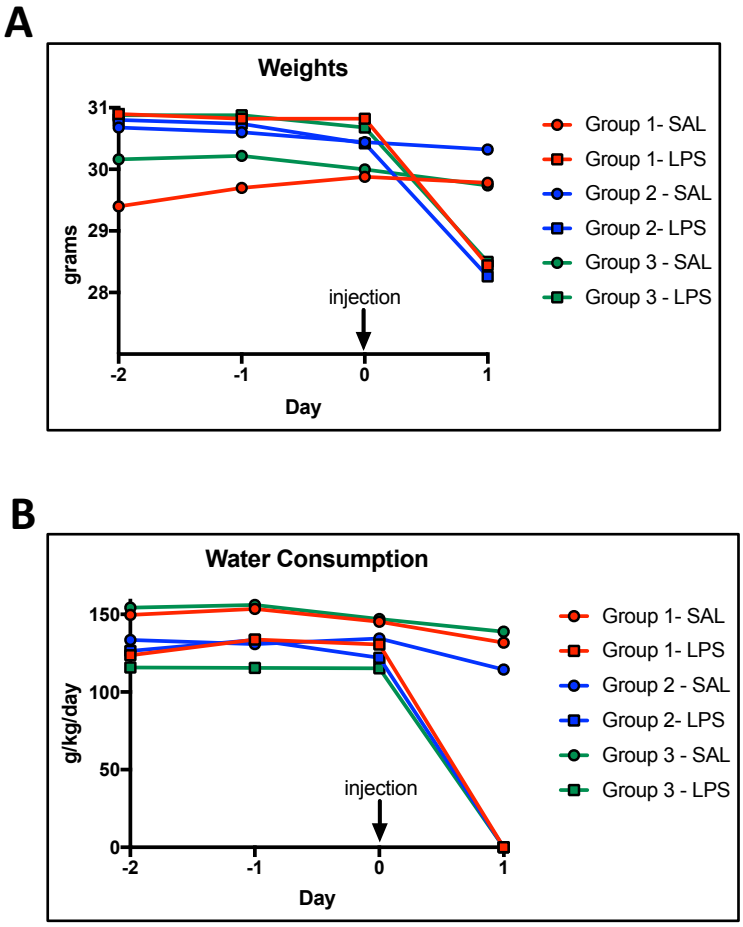


Figure 3.3: Weights and water consumption after LPS

Data verifying the effect of LPS treatment. **A.** All 3 LPS groups showed decreased weight following injection, data points are averages of 5 mice. **B.** All 3 LPS groups decreased water consumption following LPS injection, data points are averages of 5 mice.

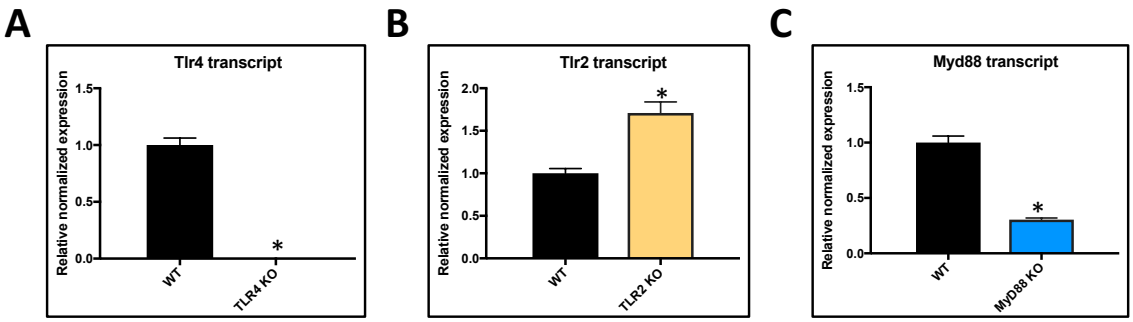


Figure 3.4: Knockout mouse qPCR

RT-qPCR on knockout mouse brain compared to wild type (C57/Bl6J). **A.** TLR4 knockout tissue showed no *Tlr4* mRNA expression. **B.** TLR2 knockout tissue showed an increase in *Tlr2* expression. **C.** MyD88 knockout tissue showed a decrease in *Myd88* expression. * is p value < 0.05, 2-tailed t-test, n =10 per group.

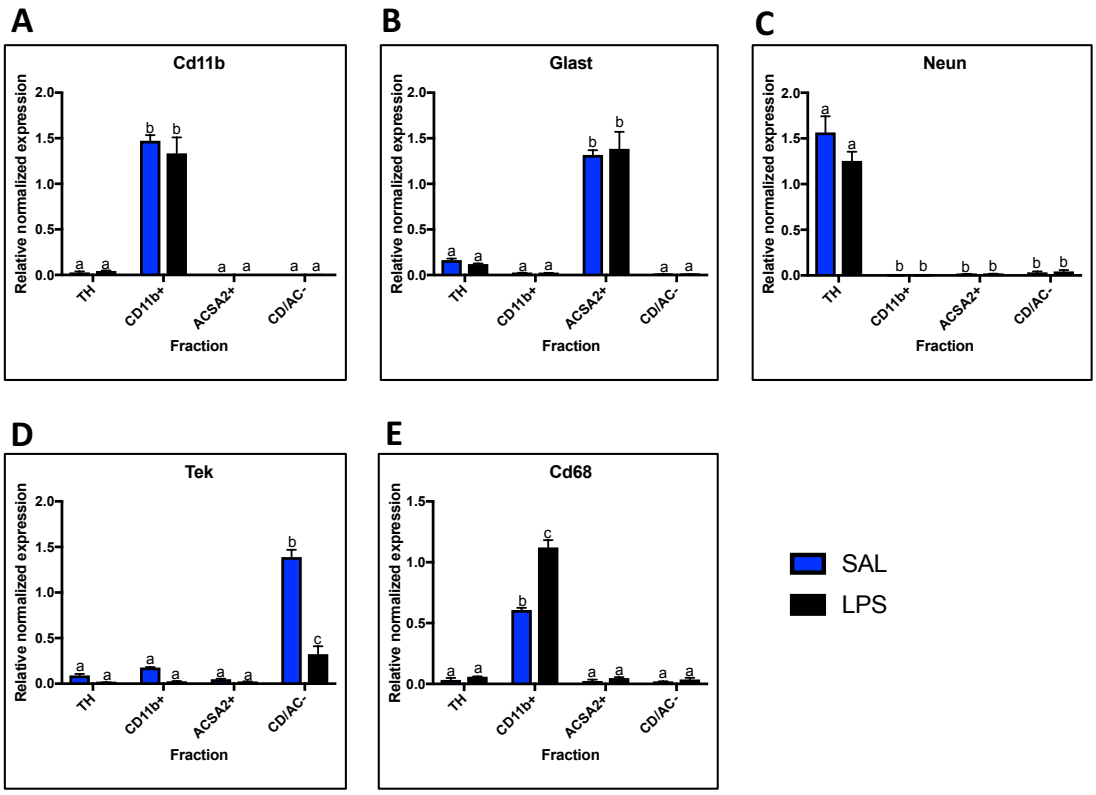


Figure 3.5: Cell-type marker mRNA expression

qPCR analysis of cell-type marker expression in the four fractions. **A.** The microglial fraction was highly enriched for the microglial marker *Cd11b* and *Cd11b* was absent in the astrocyte and negative fraction. **B.** The astrocyte fraction was highly enriched for the astrocyte marker *Glast,* and expression of *Glast* was extremely low or absent in the microglial and negative fractions. **C.** The total homogenate had high expression of the neuronal marker *Neun*. *Neun* was absent from the microglial and astrocytes fractions and was expressed in low levels in the negative fraction. **D.** The endothelial cell marker *Tek* was highly expressed in the negative fraction and lowly expressed in the other three fractions. *Tek* expression decreased with LPS in the negative fraction. **E.** The activated microglial marker *Cd68* was highly expressed in the microglial fraction, and lowly expressed in the other fractions. *Cd68* expression increased with LPS in the microglial fraction. 2 bars with the same letter are not statistically different, 2 bars with no letter in common are statistically different (2-way ANOVA with Tukey's test for multiple comparisons, $p < .05$).

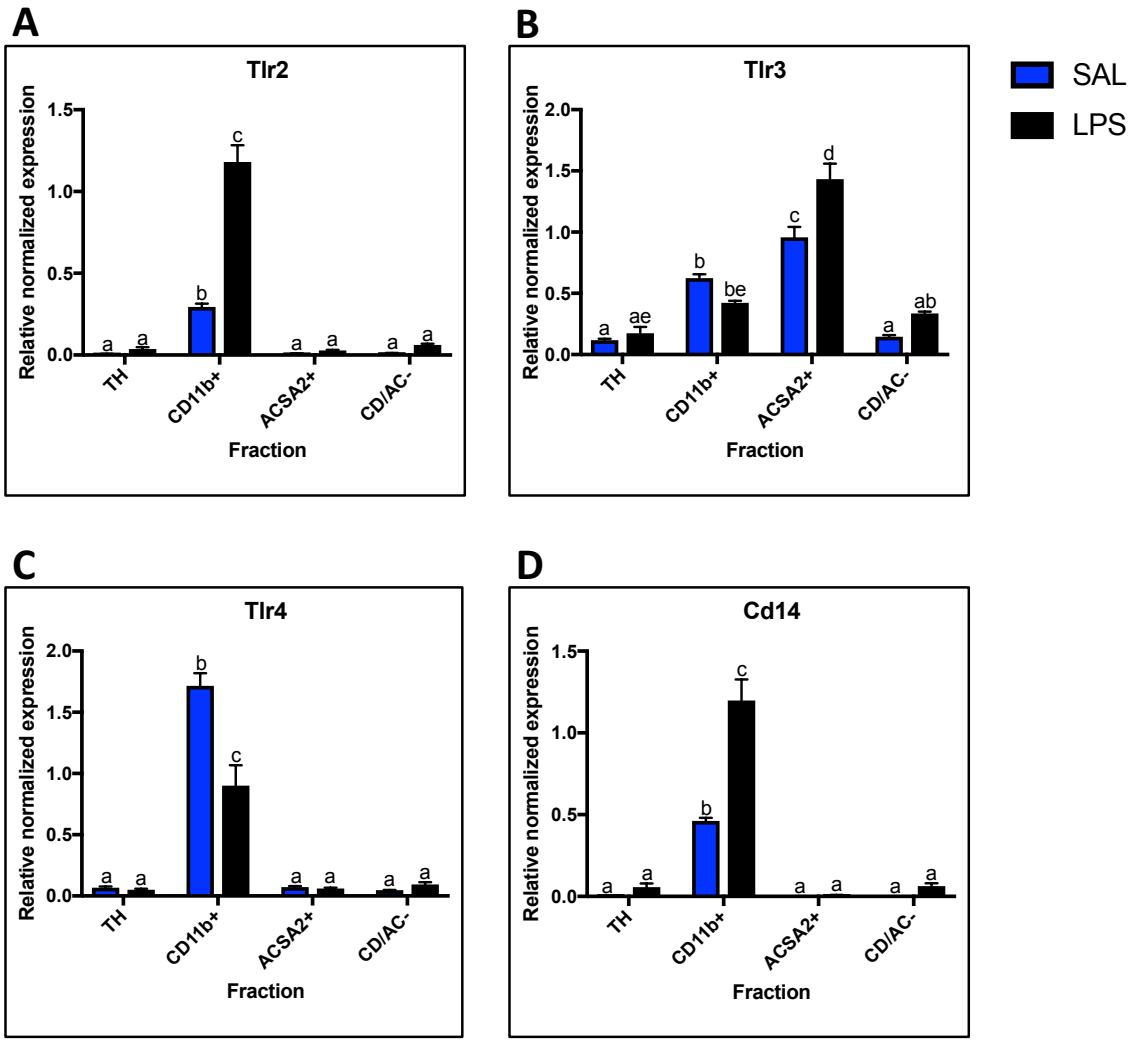


Figure 3.6: TLR mRNA expression

Fraction localization and LPS expression changes for Toll-like receptors and co-receptors measured by qPCR. **A.** *Tlr2* is expressed primarily in the microglial fraction and expression increases with LPS. **B.** *Tlr3* is enriched in both microglia and astrocytes compared to the TH, with higher expression in astrocytes. Astrocyte *Tlr3* expression increased with LPS. **C.** *Tlr4* expression is highly microglial and decreases following LPS. **D.** *Cd14* is highly enriched in microglia and increases with LPS. 2 bars with the same letter are not statistically different, 2 bars with no letter in common are statistically different (2-way ANOVA with Tukey's test for multiple comparisons, $p < .05$).

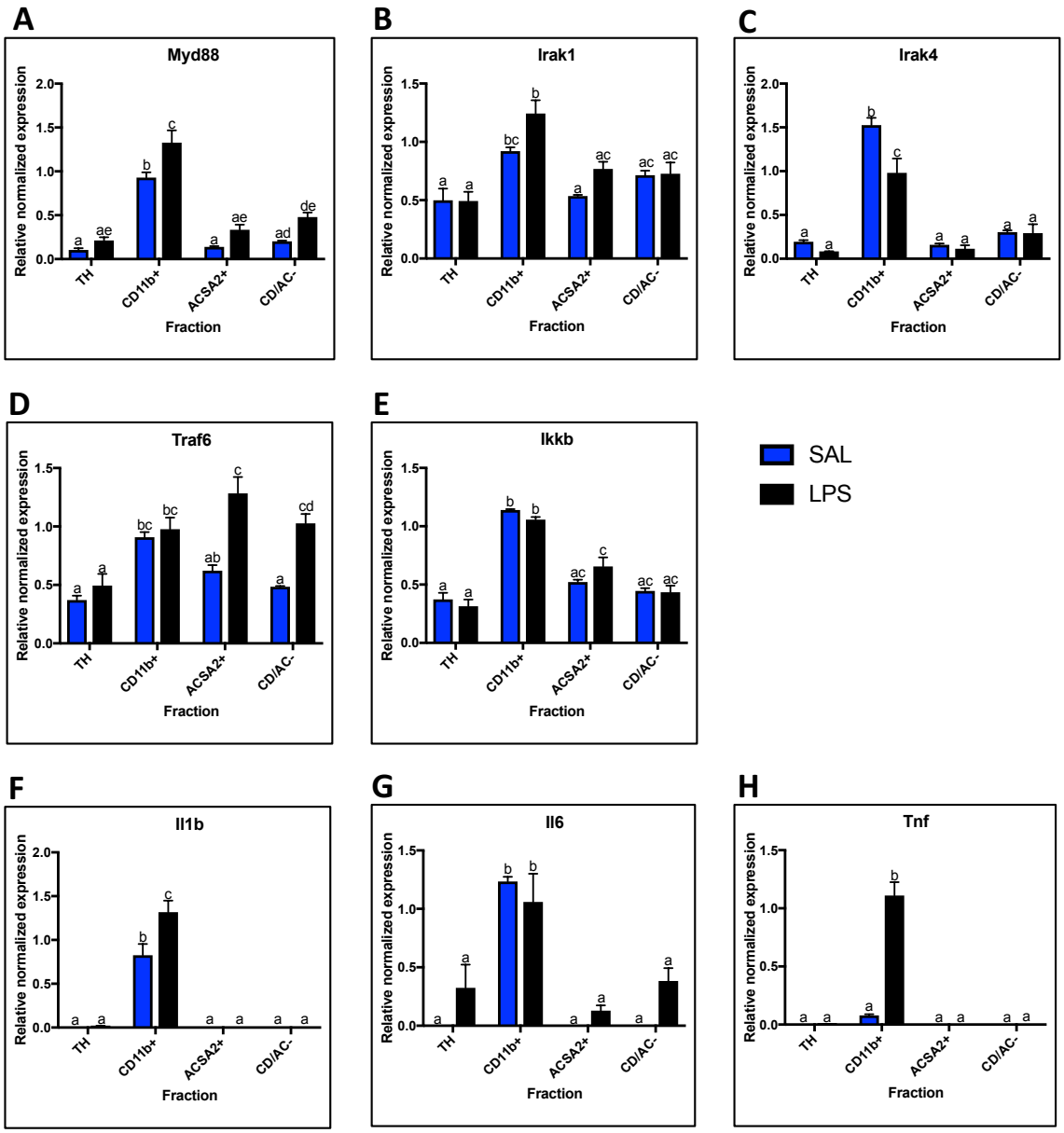


Figure 3.7: MyD88-dependent pathway mRNA expression

Figure 3.7: Continued

Fraction localization and LPS expression changes for components and outputs of the MyD88-Dependent Pathway, measured by qPCR. **A.** *MyD88* is highest enriched in the microglial fraction and increases with LPS. **B.** *Irak*1 is highest enriched in the microglial fraction, but present in moderate levels (expression is 50% or more than that of microglia) in all other fractions. *Irak1* expression increases in microglia with LPS. **C.** *Irak4* expression is highly enriched in microglia under basal conditions, and decreases in microglia after LPS. **D.** With saline, *Traf6* is enriched in the microglial fraction, but present in moderate levels in all other fractions. With LPS, *Traf6* expression increases in the astrocyte fraction and the negative fraction. **E.** *Ikkb* expression was highest in microglia, but expressed in moderate levels in all other fractions. No significant expression changes were seen after LPS treatment. **F.** Expression of *Il1b* is only detected in microglia and increases with LPS. **G.** Expression of *Il6* is only detected in microglia with saline, but is detected in all other fractions after LPS. **H.** *Tnf* was only detected in the microglial fraction and increased following LPS. 2 bars with the same letter are not statistically different, 2 bars with no letter in common are statistically different (2-way ANOVA with Tukey's test for multiple comparisons, $p < .05$).

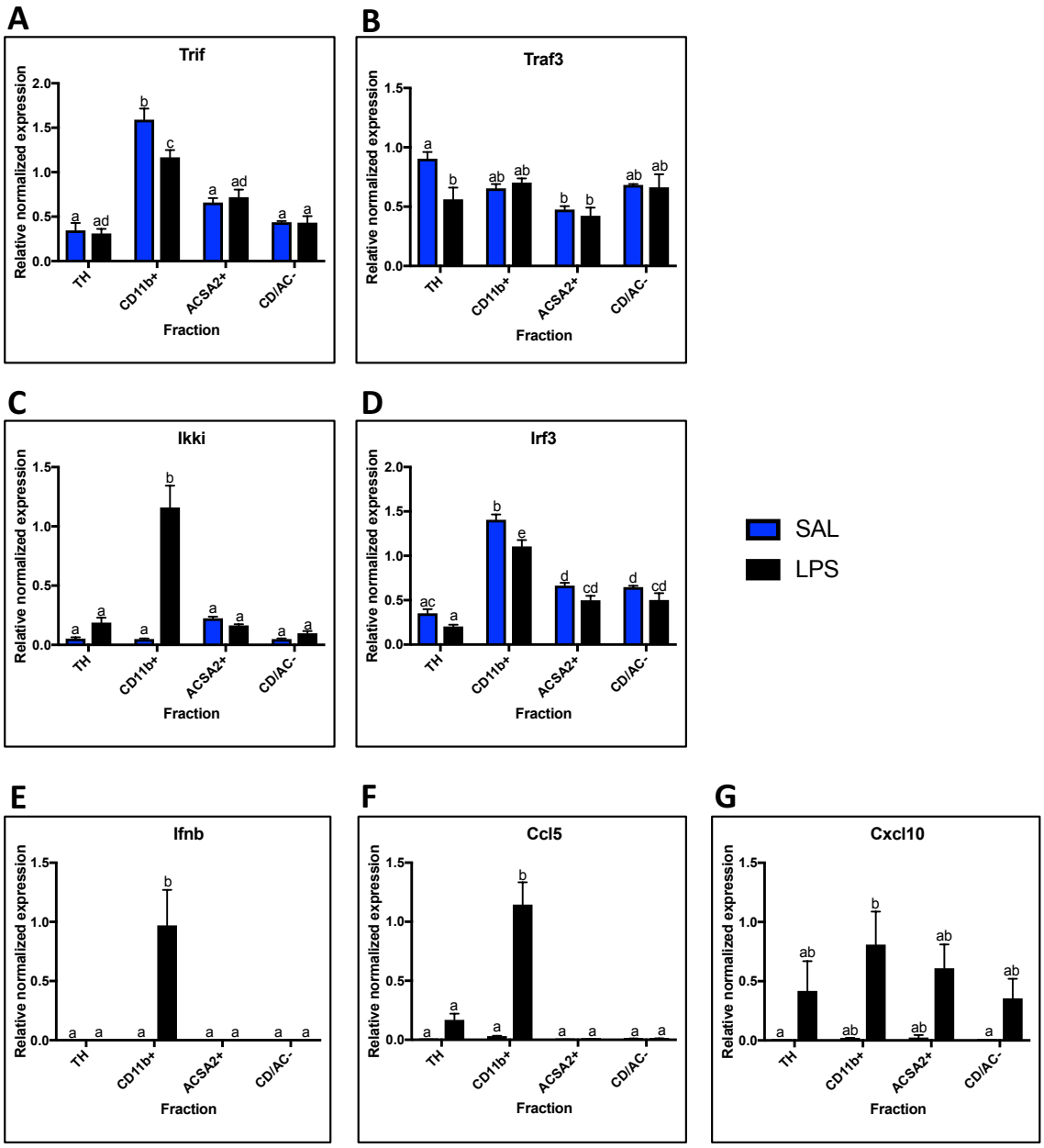


Figure 3.8: TRIF-dependent pathway mRNA expression

Figure 3.8: Continued

Fraction mRNA localization for components and outputs of the TRIF-Dependent Pathway in saline and LPS treated animals. **A.** *Trif* was highest expressed in the microglial fraction with saline and decreased with LPS. **B.** *Traf3* expression was relatively even across the fractions, with only significant difference being between the TH and the astrocyte fraction. There were no significant changes with LPS. **C.** *Ikki* expression was not significantly enriched in any fraction with saline, but was highest in astrocytes. With LPS, expression increased in the microglial fraction. **D.** *Irf3* expression was highest in the microglial fraction, but was also significantly enriched over the TH in the astrocyte fraction and negative fraction. **E.** *Ifnb* was not detected in any fractions with saline, but was expressed in microglia with LPS. **F.** Expression of *Ccl5* was expressed in low amounts in microglia with saline, but was detected in the TH with LPS and increased in microglia. **G.** *Cxcl10* was expressed in low levels in the microglial and astrocyte fractions with saline, but was detected in all fractions with LPS, although none of the changes were significant. 2 bars with the same letter are not statistically different, 2 bars with no letter in common are statistically different (2-way ANOVA with Tukey's test for multiple comparisons, $p < .05$).

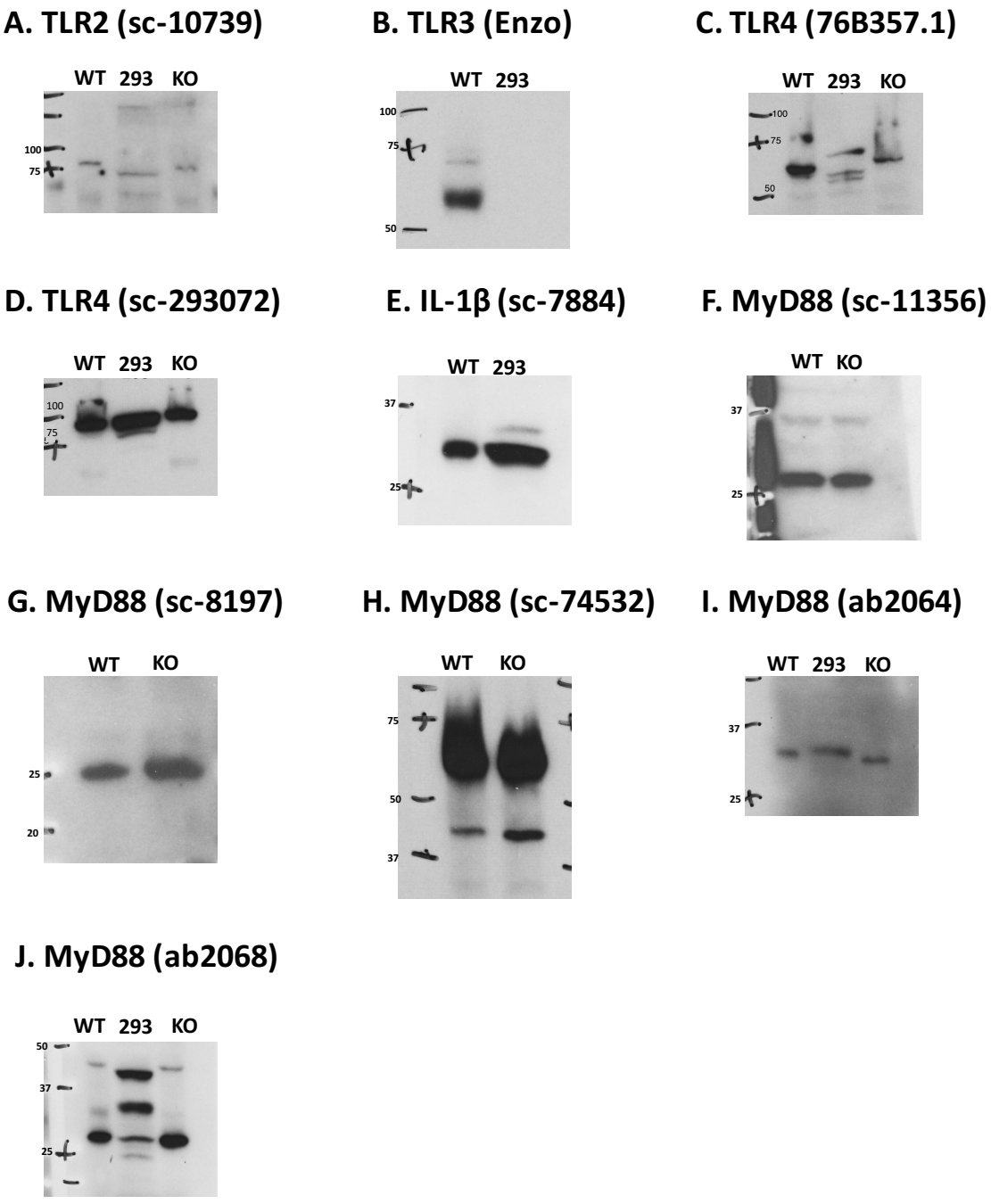


Figure 3.9: Antibody validation

Figure 3.9: Continued

Antibody tests in negative controls. **A.** The TLR2 antibody produces a signal in HEK-293 cells and TLR knockout tissue, neither of which should express TLR2. **B.** The TLR3 antibody only produced signal in the WT tissue**. C/D:** Both TLR4 antibodies produced signal in the HEK-293 cells and the TLR4 knockout tissue, neither of which should express TLR4. **E.** The IL-1β antibody produced a signal in the HEK-293 cells, which should not express IL-1β. **F-J.** All five MyD88 antibodies produced a signal in the MyD88 knockout tissue.

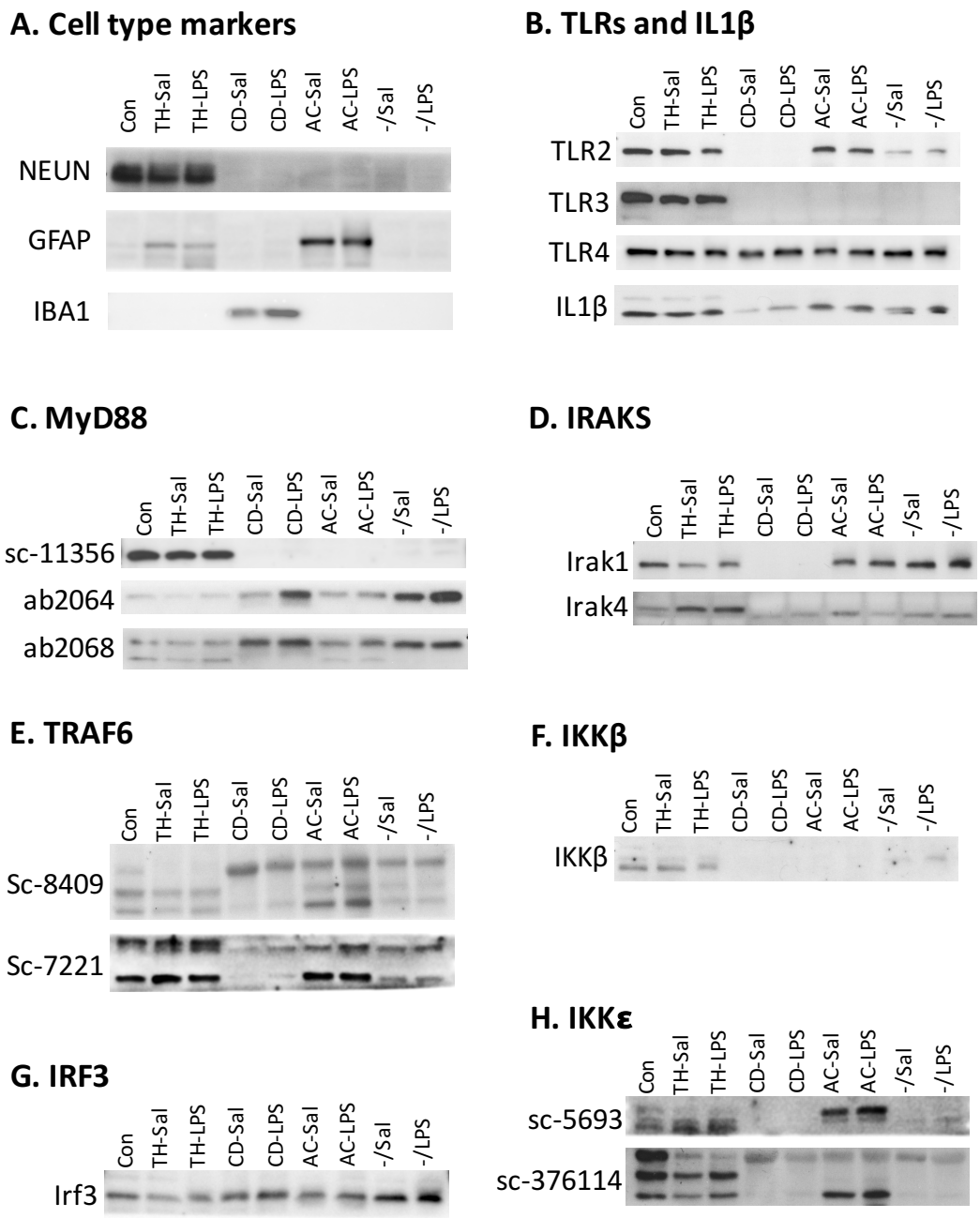


Figure 3.10: Protein expression in fractions

Figure 3.10: Continued

Protein measurement in fraction western blots. **A.** Cell-type specific antibodies verify cell-type enrichment in the fractions. NEUN, a neuronal marker, is expressed in the control sample and the total homogenate. GFAP, an astrocytic marker, is expressed in low levels in the TH and higher levels in astrocytes. IBA1, a microglial maker, is expressed in microglia. **B.** Expression for TLR2 appears to be in all fractions except microglia, while TLR3 is only detected in the TH, and TLR4 and IL-1β are detected in all fractions **C.** Blotting with 3 different MyD88 antibodies produced different results. Sc-11356 suggested MyD88 is only expressed in the total homogenate, while ab2064 and ab2068 show expression in all fractions, with highest expression in microglia and the negative fraction. **D.** IRAK1 shows expression in all fractions except microglia and IRAK4 shows expression in all fractions, but highest expression in the TH. **E.** Two different TRAF6 antibodies produce multiple bands and different results. Based on predicted molecular weight, both antibodies show highest expression in the TH and lowest expression in microglia. **F.** IKKβ showed expression in the TH and light expression in the negative fraction. **G.** IRF3 was detected in all fractions, but highest in the negative fraction. **H.** Two antibodies were used to evaluate IKKε. Sc-5693 gave signal only in the TH while Sc-376114 produced signal in all cell types.

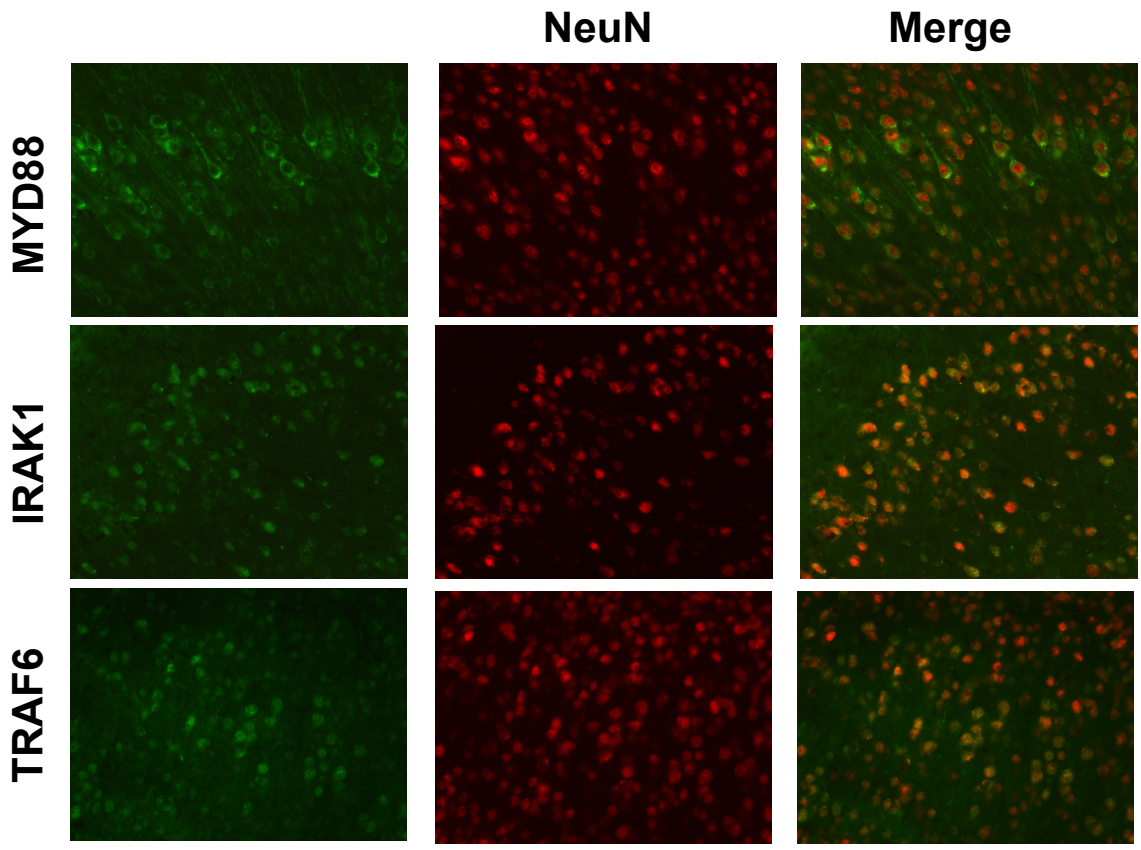


Figure 3.11: Immunohistochemistry for MyD88, IRAK1, and TRAF6

Immunohistochemistry evaluation of MyD88, IRAK1, and TRAF6 expression in the mouse cortex revealed co-localization with the neuronal maker NEUN.

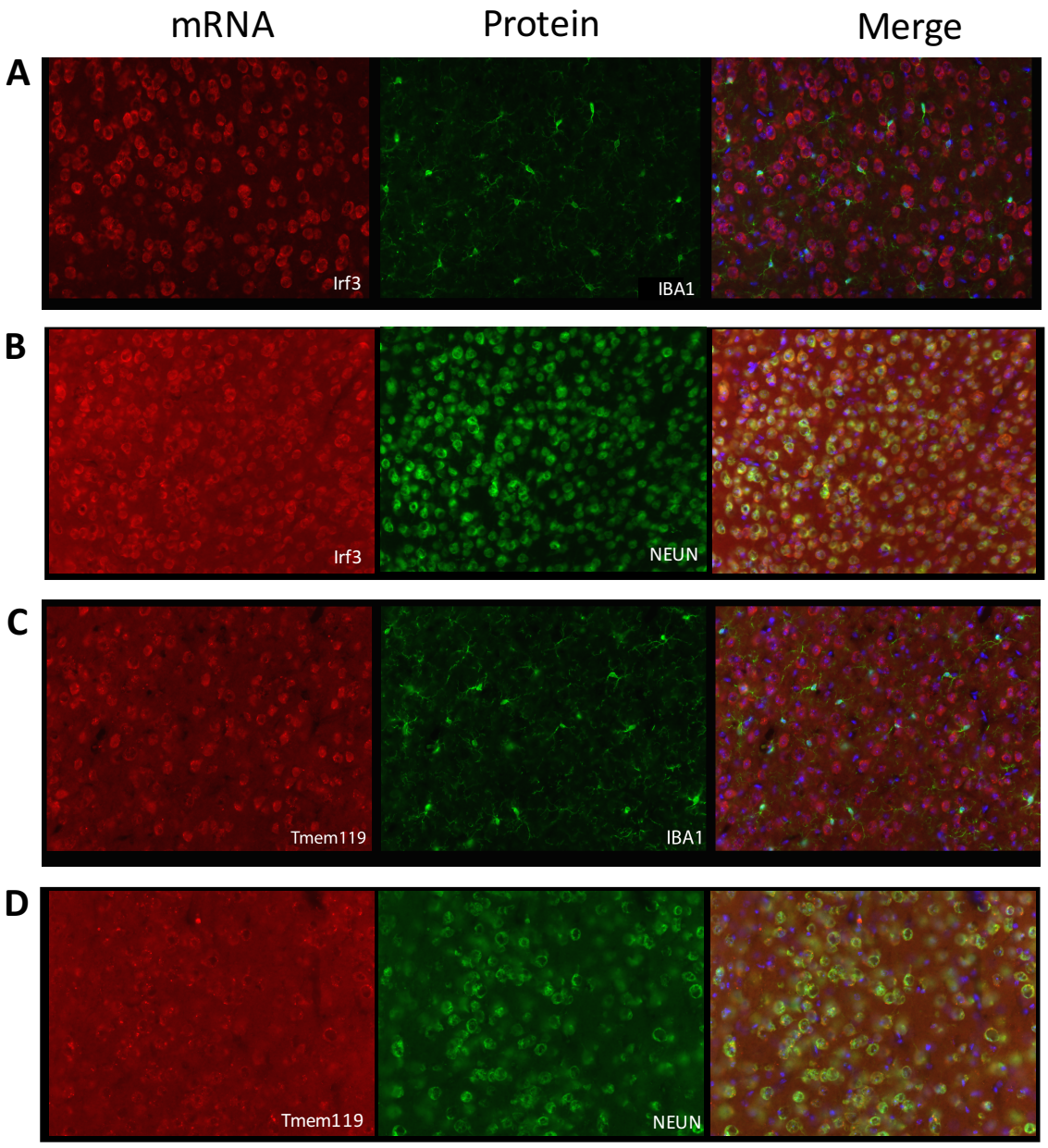


Figure 3.12: In situ hybridization for Irf3 and microglial marker

In situ hybridization compared mRNA expression with cell-type markers. **A.** *Irf3* mRNA shows little overlap with the microglial marker IBA1. **B.** *Irf3* mRNA shows high overlap with the neuronal marker NEUN. **C.** *Tmem119,* a microglial marker, shows little overlap with IBA1. **D.** *Tmem119* shows high overlap with the neuronal marker NEUN.

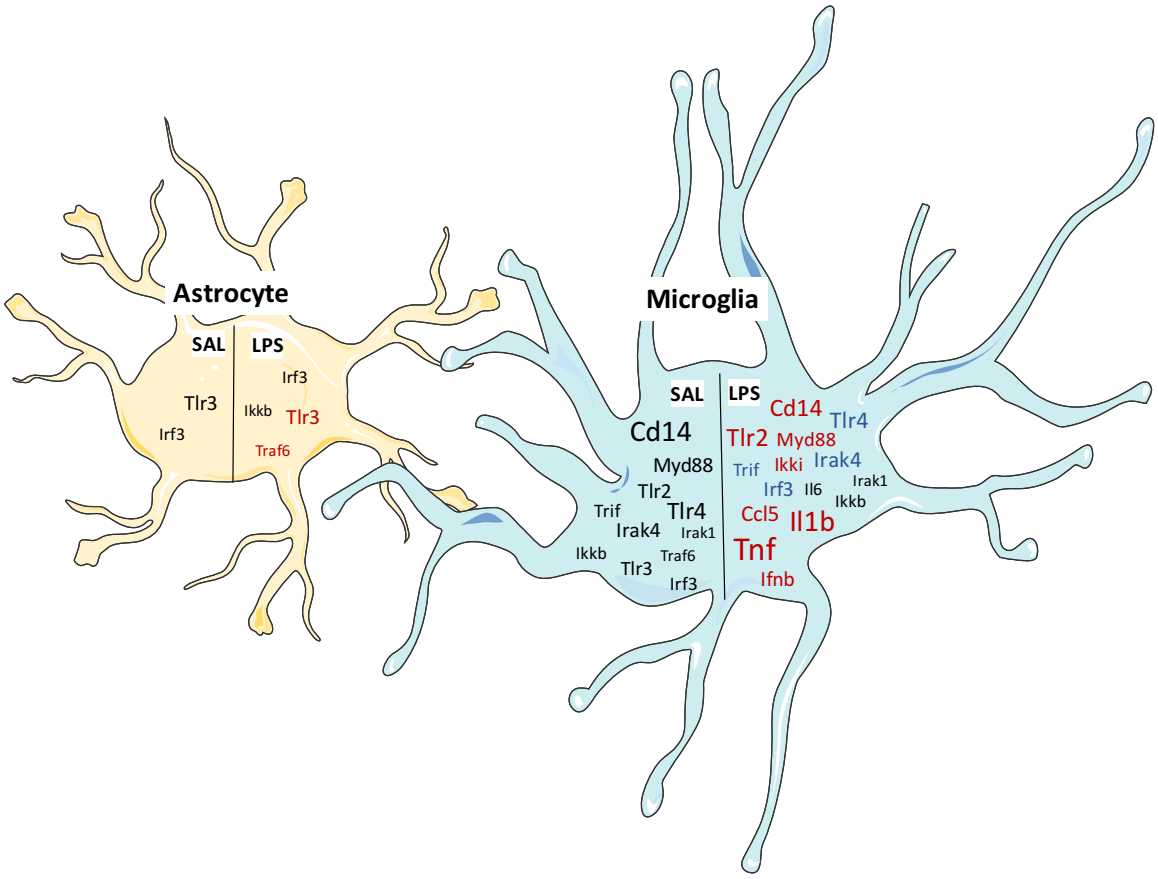


Figure 3.13: Summary of mRNA enrichment and LPS response

Microglial and astrocyte cell-type enrichment (compared to TH) is shown for TLR pathway genes in saline and LPS treated mice. The font size of each gene indicates fold-enrichment, with larger sizes meaning larger fold-enrichment. Colors on the LPS side denote whether that gene changed in that cell type with LPS treatment. Red indicates increased gene expression while blue denotes decreased gene expression.
Figure created using http://servier.com/Powerpoint-image-bank

3.VIII. TABLES

Gene Name	Gene Expression Assay
18s	4333760T
Ccl5	Mm01302427_m1
Cd14	Mm00438094_g1
Cd68	Mm03047340_m1
Cxcl10	Mm00445235_m1
IFNb	Mm00439552_s1
Ikbkb (Ikkb)	Mm01222247_m1
Ikbke (Ikke)	Mm00444862_m1
Il1b	Mm00434228_m1
Il6	Mm00446190_m1
Irak1	Mm0119538_m1
Irak4	Mm00459443_m1
Irf3	Mm00516784_m1
Itgam (Cd11b)	Mm0434455_m1
Myd88	Mm00440338_m1
Rbfox3 (Neun)	Mm01248781_m1
Slc1a3 (Glast)	Mm00600697_m1
Tek	Mm00443243_m1
Ticam1 (Trif)	Mm00844508_s1
Tlr2	Mm00442346_m1
Tlr3	Mm01207404_m1
Tlr4	Mm00445273_m1
Tnf	Mm00443258_m1
Traf3	Mm00495752_m1
Traf6	Mm0493836_m1

Table 3.1: Taqman gene expression assays used for RT-qPCR

Primary Antibodies for Westerns						
Antibody	**Manufacturer**	**Catalog #**	**Host**	**Dilution**	**Diluant**	**predicted MW**
GFAP	Invitrogen	18-0063	Rabbit	1:5000	5% NFDM	50 kD
Iba1	Wako	019-19741	Rabbit	1:1000	5% NFDM	17 kD
IKKi (M-17)	Santa Cruz	sc-5693	Goat	1:200	5% NFDM	80 kD
IKKβ (P-20)	Santa Cruz	sc-34673	Goat	1:200	5% NFDM	87 kD
IL-1β	Santa Cruz	sc-7884	Rabbit	1:200	5% NFDM	31 kD
IRAK-1 (F-4)	Santa Cruz	sc-5288	Mouse	1:200	5% NFDM	80 kD
IRF-3 (FL-425)	Santa Cruz	sc-9082	Rabbit	1:200	5% NFDM	50 kD
MYD88 (HFL-296)	Santa Cruz	sc-11356	Rabbit	1:200	5% BSA	33 kD
TLR2 (H-175)	Santa Cruz	sc-10739	Rabbit	1:200	5% NFDM	90-100 kD
TLR4 (25)	Santa Cruz	sc-293072	Mouse	1:200	5% NFDM	95/120 kD
TRAF6 (H-274)	Santa Cruz	sc-7221	Rabbit	1:200	5% NFDM	60 kD
TLR3	Enzo	ALX-804-362-C100	Mouse	1:1000	5% NFDM	117 kD
TLR4	Novus	76B357.1	Mouse	1:500	5% NFDM	95 kD
MyD88 (F-19)	Santa Cruz	sc-8197	Goat	1:200	5% NFDM	33 kD
MyD88 (E-11)	Santa Cruz	sc-74532	Mouse	1:200	5% NFDM	33 kD
MyD88	Abcam	Ab2064	Rabbit	1:1000	5% NFDM	33 kD
MyD88	Abcam	Ab2068	Rabbit	1:1000	5% NFDM	33 kD
NeuN	Abcam	ab177487	Rabbit	1:10,000	5% NFDM	34 kD
Traf6 (D10)	Santa Cruz	sc-8409	Mouse	1:200	5% NFDM	60 kD
IRAK4	Cell signaling	4363	Rabbit	1:1000	5% BSA	55 kD
IKKI (A-11)	Santa Cruz	sc-376114	Mouse	1:200	5% NFDM	80 kD
Secondary Antibodies for Westerns						
Antibody	**Manufacturer**	**Catalog #**	**Dilution**			
goat anti-rabbit IgG-HRP	Santa Cruz	sc-2301	1:1000			
goat anti-mouse IgG-HRP	Santa Cruz	sc-2302	1:1000			
donkey anti-goat IgG-HRP	Santa Cruz	sc-2020	1:1000			

Table 3.2: Antibodies used for western blots

A

Primary Antibodies for Immunohistochemistry					
Antibody	**Manufacturer**	**Catalog #**	**Host**	**Dilution**	**Diluant**
IRAK-1 (H-273)	Santa Cruz	sc-7883	Rabbit	1:50	2% Goat Serum
MyD88 (F-19)	Santa Cruz	sc-8197	Goat	1:250	2% Donkey Serum
TRAF6 (H-274)	Santa Cruz	sc-7221	Rabbit	1:50	2% Goat Serum
NEUN	Millipore	MAB377	Mouse	1:500	2% Goat/Donkey Serum
Secondary Antibodies for Immunohistochemistry					
Antibody	**Manufacturer**	**Catalog #**	**Dilution**		
Alexa Fluor 568 donkey anti-goat	Thermo Scientific	A-11057	1:1000		
Alexa Fluor 488 donkey anti-mouse	Thermo Scientific	A-21202	1:1000		
Alexa Fluor 594 goat anti-mouse	Thermo Scientific	A-11042	1:1000		
Alexa Fluor 488 goat anti-rabbit	Thermo Scientific	A-11034	1:1000		

B

mRNA	Custom Probe Sequence	Probe Label	Probe Concentration	Probe Hybridization Temperature	Primary antibody and dilution	Secondary antibody and dilution
Irf3	AGA CTG AGC CTT GTA GAA TAA	5' and 3' DIG	100nM	54°C	Anti-DIG; 1:500 (Roche # 11 333 089 001)	Donkey anti sheep (594); 1:1000 (Life Technologies #)
Tmem119		5' and 3' DIG	120nM	54°C	Anti-DIG; 1:500 (Roche # 11 333 089 001)	Donkey anti sheep (594); 1:1000 (Life Technologies #)

C

Protein	Primary antibody and dilution	Secondary antibody and dilution
Iba1	Anti-IBA1; 1:1000 (WAKO)	Donkey anti rabbit (488); 1:1000 (Life Technologies #)
NeuN	Anti-NeuN; 1:1000 (Millipore #MAB377)	Donkey anti mouse (488); 1:1000 (Life Technologies #)

Table 3.3: IHC and in situ antibodies/probes

A. Antibodies used for immunohistochemistry in figure 3.10. **B.** Probes used for in situ hybridization in figure 3.11. **C.** Antibodies used for in situ hybridization in figure 3.11.

gene	primary localization with SAL	fold enrichment over TH (SAL)	primary localization with LPS	fold change over TH (LPS)	change in TH with LPS	change in CD with LPS	change in AC with LPS	change in CD/AC- with lps
Toll-like Receptors and CD14								
Tlr2	Cd11b+	8	Cd11b+	32		↑ 4.0		
Tlr3	Acsa2+	8	Acsa2+	8			↑ 1.5	
	Cd11b+	5						
Tlr4	Cd11b+	25	Cd11b+	18		↓0.53		
Cd14	Cd11b+	75	Cd11b+	21		↑ 2.6		
MyD88-Dependent Pathway								
Myd88	Cd11b+	9	Cd11b+	6		↑ 1.4		
Irak1	Cd11b+	2.5	Cd11b+	2.5				
Irak4	Cd11b+	8	Cd11b+	12		↓0.64		
Traf6	Cd11b+	2.5	Acsa2+	2.5			↑ 2.1	↑ 2.1
			CD/AC-	2				
Ikkb	Cd11b+	3	Cd11b+	3				
			Acsa2+	2				
Il1b	Cd11b+	not detected in TH	Cd11b+	90		↑ 1.6		
Il6	Cd11b+	not detected in TH	Cd11b+	3.3				
Tnf	none significant		Cd11b+	277		↑ 13.7		
TRIF-Dependent Pathway								
Trif	Cd11b+	5	Cd11b+	4		↓0.73		
Traf3	none significant				↓0.62			
Ikki	none significant		Cd11b+	6		↑ 23.5		
Irf3	Cd11b+	4	Cd11b+	7		↓.79		
	Acsa2+	3	Acsa2+	2.5				
	CD/AC-	2	CD/AC-	2.5				
Ifnb	none significant		Cd11b+	not detected in TH		only detected with LPS		
Ccl5	none significant		Cd11b+	13		↑ 36.5		
Cxcl10	none significant							

Table 3.4: Summary of qPCR data

Table 3.4: Continued

Summary of 24 hour LPS qPCR data. Colors indicate fraction, with the microglial fraction being shown in teal, the astrocyte fraction in yellow, and the negative fraction in orange. Primary localization with SAL is determined by fraction enrichment compared to the total homogenate under saline conditions, with fold-enrichment shown in the next column. Primary localization with LPS is determined by fraction enrichment compared to the total homogenate with LPS treatment, with fold change shown in the next column. Change in each fraction with LPS is determined by comparing expression in that fraction with saline to expression in that fraction with LPS, with direction and fold-change noted. Red indicates increased expression while blue indicates decreased expression. Only significant differences are noted ($p<.05$, 2-way ANOVA with Tukey's multiple comparisons test).

Chapter 4: Microglia-specific transcriptome changes following chronic alcohol consumption

Gizelle M. McCarthy, Sean P. Farris, Yuri A. Blednov, R. Adron Harris, and R. Dayne Mayfield

4. I. ABSTRACT

Microglia are fundamentally important immune cells within the central nervous system (CNS) that respond to environmental challenges to maintain normal physiological processes. Alterations in steady-state cellular function and over-activation of microglia can facilitate the initiation and progression of neuropathological conditions such as Alzheimer's disease, Multiple Sclerosis, and Major Depressive Disorder. Alcohol consumption disrupts signaling pathways including both innate and adaptive immune responses that are necessary for CNS homeostasis. Coordinate expression of these genes is not ascertained from an admixture of CNS cell-types, underscoring the importance of examining isolated cellular populations to reveal systematic gene expression changes arising from mature microglia. Unbiased RNA-Seq profiling was used to identify gene expression changes in isolated prefrontal cortical microglia in response to recurring bouts of voluntary alcohol drinking behavior. The voluntary ethanol paradigm utilizes long-term consumption ethanol that results in escalation and models cortical plasticity seen in humans. Gene coexpression analysis identified a coordinately regulated group of genes,

unique to microglia, that collectively are associated with alcohol consumption. Genes within this group are involved in toll-like receptor signaling and transforming growth factor beta signaling. Network connectivity of this group identified *Siglech* as a putative hub gene and highlighted the potential importance of proteases in the microglial response to chronic ethanol. In conclusion, we identified a distinctive microglial gene expression signature for neuroimmune responses related to alcohol consumption that provides valuable insight into microglia-specific changes underlying the development of substance abuse, and possibly other CNS disorders.

4.II. INTRODUCTION

Microglia comprise 5% of all cells present within the adult brain (Lawson et al. 1990) and serve as the principal cell type responsible for innate and adaptive immune processes. Mature microglia change morphological and expression profiles to participate in CNS homeostasis and respond to pathological insults.

Acute and chronic substance abuse cause persistent changes in gene expression, which can cause functional changes that contribute to maladaptive behavior (Nestler et al. 1993; Kerns et al. 2005; Piechota et al. 2010). Neurogenomic studies for substance abuse have primarily utilized whole tissue homogenates, representing a mixture of cell-types, including neurons, astrocytes, oligodendrocytes, and microglia. These cell types express many common genes; however, they each have a unique transcriptional landscape (Cahoy et al. 2008; Y. Zhang et al. 2014). While studying the gene expression changes of whole

tissue is important to understand communication within a cellular ensemble, this approach may fail to discern specific cellular responses occurring within an individual cell-type (Lacagnina et al. 2016). Isolated microglial cells have been shown to undergo a unique set of gene expression changes associated with aging, neurodegeneration, and depression (Chiu et al. 2013; Gonzalez-Pena et al. 2016; Hickman et al. 2013; M. D. Li et al. 2015; Vincenti et al. 2016; Orre et al. 2014). Exposure to *in vitro* alcohol leads to microglial activation and the release of specific proinflammatory cytokines, while long-term alcohol consumption results in microglial activation in rodent models and human postmortem brain (He & Crews 2008; Fernandez-Lizarbe et al. 2009). These studies have led to the idea that alcohol exposure leads to an inflammatory state which further drives ethanol consumption. We hypothesize that microglial gene expression represents an important molecular phenotype correlated with alcohol consumption, and that characterizing the transcriptome of microglia versus total cell populations will provide a framework for the identification of specific alcohol-induced alterations in neuroimmune gene expression.

The prefrontal cortex (CTX) is an important brain region involved in alcohol use disorders (AUDs), serving as a key link in both positive and negative affect associated with substance abuse (Koob & Volkow 2010). Alcohol exposure triggers a cascade of immune-related gene expression changes within adolescent and adult CTX (Osterndorff-Kahanek et al. 2013) suggesting that microglia may be critical mediators underlying the transition from abuse to dependence. We utilized RNA-sequencing to profile the transcriptome of isolated microglia in response to voluntary alcohol consumption and

identified coordinated gene expression changes that were specific to the microglial cell population. These findings support the hypothesis that neuroimmune genes underlie the progression of alcohol use to AUDs.

4.III. Materials and Methods

4.III.a. Ethics Statement

All procedures were approved by the University of Texas at Austin Institutional Animal Care and Use Committee (animal protocol number AUP-2013-00061) and adhered to the NIH Guidelines. The University of Texas at Austin animal facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

4.III.b. Animals and voluntary ethanol consumption

Studies were conducted in adult (6-8 weeks old) C57BL/6J (B6) male mice (Jackson Laboratories, Bar Harbor, ME). Mice were individually housed and allowed to acclimate to upright bottles one week before the start of the experiment. The experimental rooms were maintained at an ambient temperature of 21±1°C, 40-60% humidity on a regular light/dark schedule, with *ad libitum access to* food and water.

An every-other-day 2-bottle choice (EOD-2BC) paradigm (Osterndorff-Kahanek et al. 2013) was used with mice randomly assigned to control or alcohol-consumption treatment groups. Control and treatment groups each contained 12 mice per group (24 total). Control animals had daily access to water while treatment groups had every-other-

day access to both water and a 15% (v/v) alcohol solution (access to water only on non-treatment days). An empty cage control was used to account for spillage. Alcohol bottle positions were alternated at each exposure to control for potential side preferences. Alcohol and water bottles were weighed on treatment days, and animals were weighed once per week to calculate consumption. The study concluded after 60 total days (30 drinking days) and blood alcohol concentrations were determined prior to sacrifice.

4.III.c. Tissue harvest and microglial isolation

Mice were sacrificed immediately after the final alcohol drinking session and anaesthetized using isoflurane followed by transcardial perfusion with PBS. The CTX was dissected as previously described (Osterndorff-Kahanek et al. 2013) and put into cold Hank's Balanced Salt Solution (HBSS). Microglial isolations (Nikodemova & Watters 2012) were performed by first mincing individual CTX tissue on ice and suspending in cold HBSS. Approximately 1% of the minced tissue mixture per sample was taken as a total homogenate sample (includes all cell types). Homogenates were centrifuged at 1000 x g for 10 minutes at 4°C. The supernatant was removed and the cells were flash frozen in liquid nitrogen. The remaining minced tissue was used for microglial isolation with magnetic bead sorting technology. This method was chosen because it is shown to preserve cellular phenotype and RNA integrity as well as result in high purity compared to other methods (Nikodemova & Watters 2012; Ju et al. 2015). Briefly, tissue solution was manually dissociated using the Neural Tissue Dissociation Kit (Papain) (Miltenyi Biotec, Germany). Dissociated tissue was passed through a 70 μM strainer (Miltenyi Biotec), centrifuged at 300 x g, then resuspended in 30% percoll (Sigma-Aldrich, St. Louis, MO). The percoll-cell suspension was centrifuged at 700 x g for 15 minutes at room temperature

with the myelin fraction removed from the top of the suspended solution. Cells were incubated with Cd11b MicroBeads (Miltenyi Biotec) and eluted using MS columns to collect Cd11b+ cells. Previous studies have shown that microglial isolation using this protocol yields pure microglia and does not perturb gene expression (Nikodemova & Watters 2012) (Ju et al. 2015; de Haas et al. 2008).

4.III.d. RNA isolation and PCR validation

RNA, from individual samples, was isolated using the RNeasy Micro Kit (Qiagen, Germany). RNA concentration and quality (RNA integrity number) was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) using the Agilent RNA 6000 Pico Kit. All of the examined total homogenate and microglia samples met quality-control criterion. There were no significant different differences in RNA integrity number (RIN), evaluated based upon the 28S to 18S ratio, between total homogenate and microglia samples ($P = 0.61$). There were no significant differences in quality or quantity of microglial RNA between the control and ethanol groups. Total RNA was reverse transcribed into cDNA using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc., Rockford, IL). Applied Biosystems Taqman® Gene Expression Assay primers were used for quantitative real-time PCR (q-rtPCR). Assay ID's included Mm0434455_m1 (*Itgam*/Cd11b), Mm01253033_m1 (*Gfap*), Mm01248771_m1 (Rbfox3/Neun), Mm00443243_m1 (*Tek*), Mm00495930_m1 (*Ugt8a*), and 4333760T (*18s*). Q-rtPCR reactions were performed using SsoAdvanced™ Universal Probes Supermix (BioRad, Hercules, CA) in 10-µL

reactions containing 1 ng of cDNA. All reactions were performed in technical triplicates for each biological replicate and included a negative no-template control. Q-rtPCR reactions were carried out using the CFX384 Real-Time System (BioRad). Samples were normalized to 18s and relative expression was determined using the CFX software (BioRad). Examining the five cellular makers with Q-rtPCR verified Cd11b positive cells were enriched compared to the Cd11b negative fraction and total homogenate (**Fig. 4.6**).

4.III.e. RNA Sequencing and Bioinformatics Analysis

Samples were Poly(A) selected using the Poly(A) Purist Kit (Thermo Fisher Scientific Inc.) and prepared using NEBNext® Ultra™ Directional RNA Library Preparation Kit (New England BioLabs, Ipswich, MA). Samples were sequenced on the NextSeq 500 (Illumina, San Diego, CA) using (75 bp; paired-end reads) to a minimum target depth of 20 million reads per sample. Reads were mapped to the mouse reference genome (UCSC, mm10) using the alignment tool STAR (Dobin et al. 2013). Raw RNA-sequencing files were evaluated for quality control using FastQC (0.11.13) and one control microglial sample was excluded from further analysis due to low sample quality. HTSeq, was used for counting mapped sequencing reads. Expression profiles among individual biological replicates within each group were strongly correlated with a mean pearson's correlation coefficient of 0.97 for total homogenate (range: 0.93-0.99) and 0.95 for microglia samples (0.90-0.97). Quantified read counts were normalized and analyzed for differential expression using DESeq2 (v 1.10.1), between alcohol drinkers and water drinking controls for microglial and homogenate tissue preparations, within the R

statistical computing environment (v3.3.1)(Love et al. 2014). Statistical significance was assessed using a nominal p-value less than or equal to 0.05; this threshold was selected to balance type-1 and type-2 error rates for performing pathway and network analyses.

Normalized read counts, scaled according to the library size of individual samples, were filtered for non-replicable low-abundance measurements by removing the lowest 5% of mean expression occurring in less than 95% of samples. The ratios, of major cell-type specific microglial markers *Itgam* (Cd11b) and *Tmem119,* using reads per kilobase per million mapped reads (RPKM), were compared within and across all groups to assess potential confounds of the isolation protocol on transcriptome measurements. *Itgam* and *Tmem119* were similar between the TH and Cd11b+ fractions, with an average ratio (Cd11b+/TH) of 0.88 ± 0.11. Because Cd11b levels increase with immune activation while Tmem119 levels do not (Satoh et al. 2016; Roy et al. 2006), the ratios of these two markers remaining similar after isolation suggests that the magnetic-bead isolation did not disassociate cellular signatures. Weighted gene coexpression network analysis (WGCNA), using filtered $\log_2$ transformed expression data, was then applied to construct scale-free networks that specify coordinately regulated gene sets (i.e. modules) (Langfelder & Horvath 2008). Parameters used for WGCNA were performed in agreement with prior specifics used in our laboratory (Farris et al. 2014), including a minimum module size equal to 100 genes and a 0.99 tree-cut height. Microglial and total homogenate global gene expression correlations were each separately raised to a beta value equal to 6, reaching a common scale-free threshold above 0.75 for a signed hybrid analysis. The maximal statistical significance for microglial and total homogenate gene

set composition was determined using a fishers-exact test, corrected for multiple comparisons using a Benjamini & Hochberg false discovery rate (FDR) less than 0.05. Individual gene sets were functionally annotated using the web-based software application Enrichr (Kuleshov et al. 2016; E. Y. Chen et al. 2013). Select gene sets, based upon statistical and biological significance, were assembled to visualize network properties using the bioinformatics tool Cytoscape (v3.4.0) (Shannon et al. 2003). RNA-Seq data can be accessed under GSE91387.

4.IV. Results and Discussion

4.IV.a. Transcriptome profiles of isolated microglia and total homogenate

Transcriptome profiles of the microglia (Cd11b+) and tissue homogenate (TH) from the CTX of individual samples were sequenced to a target depth of 20 million reads. A total of 17,377 genes were reliably detected in Cd11b+ samples and 19,876 genes detected in TH. Among the two preparations 17,172 genes were commonly expressed, with 205 unique transcripts in the Cd11b+ fraction and 2,704 unique transcripts in the TH. In agreement with single-cell sequencing of major CNS cell-types (Darmanis et al. 2015), the microglial population expressed not only a fewer number of genes, but the overall expression distribution of detected transcripts was lower in magnitude (**Fig. 4.1**). Although the isolated microglial population is being compared to a cumulative admixture of all major CNS cell-types present within CTX, this blunted profile further underscores the need for examining a solitary cellular population. Mean expression of genes present

in isolated cells versus the mean expression of genes common to TH demonstrates the relative enrichment of genes more highly localized to microglia. The cellular marker Cd11b+ (*Itgam*) used for separation of the microglial fraction showed nearly 50 times higher abundance relative to TH. *Tmem119*, an additional microglia-specific marker (Satoh et al. 2016; Bennett et al. 2016), was elevated to approximately the same degree as *Itgam*; extending PCR validations (**Fig. 4.6**) and substantiating the enrichment protocol for assessing transcriptome-wide measurements derived from microglia. Consistent with the predominant function of microglia, gene ontology analysis of the top 100 microglial-enriched genes affirmed an over-representation for genes involved in mediating an 'inflammatory response' (GO:0006954) and 'cytokine receptor binding' (GO:0005126).

4.IV.b. Differential expression following chronic ethanol consumption

An every-other-day 2-bottle choice alcohol-drinking paradigm (EOD-2BC) was undertaken to evaluate gene expression changes related to chronic alcohol exposure (**Fig. 4.7**). EOD-2BC, compared to other voluntary alcohol drinking mouse models, leads to escalation of drinking over time and high alcohol consumption (Melendez 2011). Previous work has shown that EOD-2BC leads to the greatest number of neuroimmune related changes in CTX (Osterndorff-Kahanek et al. 2013); however, a portion of these changes may not have been cell-type specific. RNA-Seq of an isolated microglial population using EOD-2BC was undertaken to determine the contribution of gene expression changes within this cell-type compared to tissue homogenate. Differential

gene expression analysis for alcohol drinking exposure respectively identified 1,010 microglia and 2,461 total homogenate changes (**Fig. 4.2**). The reduced number of changes occurring in microglia is expected given the additional CNS cell-types present within the total homogenate.

Contrasting the gene expression profiles for the two preparations shows isolated microglia have a similar range in fold-changes within the CTX due to alcohol (**Fig. 4.2a**). The observed changes are less than two-fold for either microglia or the total homogenate; however, labeling the top 20 statistically significant genes shows there are definitive biological differences between the experimental conditions due to EOD-2BC alcohol consumption. Small fold-change differences in alcohol-induced gene expression, and other biological responses, are not uncommon for the CNS (Lovinger & Crabbe 2005). Chronic alcohol exposure in B6 mice evokes time-dependent change in gene expression (Osterndorff-Kahanek et al. 2015) (Smith et al. 2016), which may not achieve a chronic cumulative steady-state of induction suggested for some other drugs of abuse (Hope et al. 1994). The observed changes in gene expression may serve as a surrogate measure for cellular mechanisms activated as a result of EOD-2BC alcohol consumption, with select differences that are representative of microglial activation.

A subset of genes (164 genes in total) is regulated by EOD-2BC in both microglia and the total homogenate (**Fig. 4.2b**). Despite this mutual overlap, 44% of these changes (72 genes) occur in opposite directions. Additionally, although a small percentage of microglia are present within the total homogenate a total of 864 gene expression changes are uniquely discerned in the isolated cells. Alcohol-induced changes within microglia

affected a broad spectrum of functional categories, several of which were not enriched within the admixture of CNS cell-types. For example, alcohol-induced microglial changes were over-represented in genes attributed to 'TGF-beta receptor signaling' (GO:0007179, P = 9.08 E-05) and 'chronic inflammatory response' (GO:0002544, P = 1.57 E-03). TGF-beta is a cytokine expressed in brain, capable of controlling microglial activation and triggering neuroinflammation (Lodge & Sriram 1996). Excessive and chronic alcohol abuse modulates similar inflammatory processes in the liver (Dooley & Dijke 2012), involving TGF-beta and downstream signaling involving SMAD proteins (P = 5.21 E-04). The extent to which these systems are affected within the brain due to the alcohol abuse is largely unknown, but corresponding changes in CNS microglial may indicate their involvement may extend beyond liver dysfunction in an AUD. Importantly, these alterations in gene expression pathways witnessed in the isolated CNS microglial population are obscured within the total homogenate. The selective effects registered indicates the value of cellular separation to draw upon microglial-specific gene expression changes that take place in mouse CTX following alcohol drinking exposure.

4.IV.c. Gene coexpression networks related to chronic alcohol exposure

Coordinate expression of multiple genes enables biological function of intricate cellular systems. Weighted correlation networks, including all genes within their respective cellular populations, were evaluated to distinguish particular subsets of coordinately regulated gene expression clusters (i.e. modules). The assayed transcriptomes partitioned into 18 microglial modules (MGM) and 19 total homogenate

modules (THM) (**Fig. 4.8**). Each of the individual gene expression modules were representative of varied biological categories, demonstrating the utility of this approach for delineating transcriptome substructure. Ranking the relative conservation of MGM and THM, based upon coinciding genes, illustrates the overall similarity and dissimilarity of the two experimental factions (**Fig. 4.3**).

Altogether, there are seven MGM and THM duos sharing a significant number of common genes. Examining the top seven strongest MGM-THM pairwise relationship shows each of these shared overlapping gene sets denote a meaningful intracellular group, such as 'chromatin modification' (MGM5-THM6, P = 4.21E-03), 'mitochondrial matrix' (MGM1-THM3, P = 1.70 E-10), and 'RNA splicing' (THM16–MGM8, P = 4.60 E-12). Only one of the MGM-THM pairs, MGM5-THM6, showed evidence of chronic alcohol-responsive changes in gene expression; however, the direction of MGM5 change was opposite to THM16. Transcriptional regulation through epigenetic modification of chromatin is proposed as a major component in neuronal plasticity (Maze et al. 2015). DNA methylation alters chromatin structure, affecting persistent regulation of gene expression and CNS plasticity. Stimulation of neuronal firing leads to a redistribution of DNA methylation (J. U. Guo et al. 2011), suggesting pharmacological interactions affecting neuronal activity impacts epigenetic mechanisms of transcriptional regulation. Increased expression of genes involved in histone modification after chronic alcohol may be a surrogate indicator of epigenomic adaptations in neuronal circuits, or potentially other non-microglial cell types (Starkman et al. 2012).

EOD-2BC alcohol consumption altered the expression of seven THMs, four of which (THM19, THM15, THM13, THM12) were distinctly separate from MGMs. Alcohol-induced changes in these THM likely represent molecular processes set apart from microglial responses. THM19 is significantly decreased in EOD-2BC mice, representing an overall alcohol-driven reduction in kindred genes within CTX. THM19 is made-up of a significant number of oligodendrocytes-related genes (P = 3.31 E-03); which is the CNS cell responsible for myelinating neuronal axons. Human oligodendroglial genes are decreased in human alcoholic CTX (Lewohl et al. 2005), featuring cellular adaptations that are potentially conserved between human and mouse CTX subjected to repeated alcohol. THM19 is inversely correlated to THM15, 13, and THM12; wherein these three THM are intrinsically related with an average rank-based intermodule correlation equal to 0.88. THM15, THM13, and THM12 are collectively enriched for neuronal gene expression signatures (P = 1.72 E-05).

Gene expression for MGM5, MGM15, MGM3, and MGM13 are differentially regulated by EOD-2BC alcohol consumption. MGM15, MGM3, and MGM13 are by and large unrelated to THMs, indicating transcriptome measurements from an admixture of CNS cells may fail to notice genes affected by alcohol drinking behavior within these MGM groups. Cd11b is an integral membrane-associated protein that is a cellular marker of CNS microglia; however, Cd11b is also present on the surface of other monocyte-derived lineages, including macrophages. Macrophages are capable of invading the CNS, sparking neuroinflammation and neurodegeneration (Shemer & Jung 2015). MGM15, MGM3, and MGM13 were further assessed for unintended non-microglial cellular gene

expression arising from Cd11b+ enrichment. Expression of genes belonging to MGM13 and MGM15 were indistinguishable from other CNS or immune cell-types. The highest mean expression of MGM15 and MGM13 containing genes occurred in macrophage and stromal cellular populations (**Fig. 4.9**). MGM3 encompassing 1,135 genes, showed increased expression following EOD-2BC alcohol exposure (**Fig. 4.4**). MGM3 is significantly enriched for microglial-expressing genes involved in immune-related signaling systems (**Fig. 4.4B**). Genes within MGM3 are positively correlated with the total amount of alcohol consumed (Pearson r=0.77, $P = 0.01$). Matching the same genes within the total homogenate obscured this correlation with alcohol consumption (Pearson r=-0.04, $P = 0.88$), stressing the importance of isolating a microglial cellular population in an animal model of alcohol consumption to unveil a genomic relationship with the neuroimmune system. Although additional cell-types are capable of relaying an immune response, MGM3 expression was significantly higher in microglia compared to other major CNS or immune cell-types (**Fig. 4.4C/D**). There are several known microglia genes within MGM3, including cell-type specific markers *Tmem119*, *Cx3cr1*, *Aif1* (Iba1), suggesting MGM3 expression is mainly restricted to this particular CNS immune cell.

The top biological processes representative of MGM3 were 'activation of innate immune response' (GO:0002218) and 'toll-like receptor signaling pathway' (GO:0002224), which affect alcohol neurotoxicity and consumption (Blednov, Benavidez, et al. 2011) (Lippai et al. 2013). The MGM3 coexpression network (**Fig. 4.5**) was constructed to identify specific genes relevant to the interaction of microglia with EOD-2BC alcohol consumption. Central to MGM3 lies sialic acid binding Ig-like lectin

H (*Siglech)*. *Siglech* is a putative marker of isolated microglia (Chiu et al. 2013); which modulates type I Interferon secretion associated with microglial activation and phagocytosis of glioma cells (Blasius et al. 2006; Kopatz et al. 2013). Brain specific expression and function of Siglech has yet to be completely ascertained, however, because MGM3 shows ontologies related to TLR signaling, particularly endosomal TLRs which promote interferon secretion, it is possible that *Siglech* is regulating this response in brain. Although not all of the genes expressed within MGM3 are individually altered in relation to alcohol consumption, there is a dense interrelationship among MGM3 containing genes that may be important for the observed phenotype. Cathepsin proteases *Ctsf* and *Ctss* are examples of two MGM3 genes previously shown to causally affect multiple behavioral models of alcohol consumption (Blednov, Ponomarev, et al. 2011). The presence of *Ctsf* and *Ctss* among the most connected genes within the MGM3 network may suggest there are additional microglial-immune genes involved in alcohol consumption that are unaddressed by examining the total homogenate. For example, legumain (*Lgmn,* also known as asparagine endopeptidase or AEP), 16.5-fold enriched within microglia, is differentially expressed and correlated with total alcohol consumption in the microglial but not in total homogenate. Acting as an asparagine endopeptidase, Legumain is important for the cleavage and activation of toll-like receptors (Sepulveda et al. 2009). Legumain works in concert with cathepsins on toll-like receptors, also expressed within MGM3, to bring about optimum signaling (Ewald & Barton 2011). The joint presence of numerous cellular markers alongside genes with

shared activity in immune function asserts MGM3 is a microglia-specific gene network, which may designate a convergence of an alcohol-responsive microglial signature.

In addition to highlighting ethanol-induced changes in TLR and immune signaling, the changes in MGM3 suggest that ethanol is perturbing microglial TGF-β signaling. Specifically, looking at the differentially expressed genes within MGM3 revealed (**Table 4.1**) enrichment for transforming growth factor beta receptor signaling pathway (GO:0007179) and R-SMAD binding (GO:0070412). Recent studies have highlighted the important role of TGF-β in microglial homeostasis and in the regulation of immune response. Butovsky et al. found that *Tgfb1* was specific to microglia in the adult CNS and that treatment of cultured microglia with TGF-β1 resulted in increased expression of 60 genes, half of which were identified in MGM3 (Butovsky et al. 2013). Furthermore, CNS-TGF-β1 $^{-/-}$ mice had deficits in extracellular glutamate and synaptic plasticity, suggesting a key role for TGF-β1 in regulating these processes. Butovsky and group also looked at perturbations of microglial molecules that are involved in TGF-β signaling and were suppressed in the CNS-TGF-β1 $^{-/-}$ mice. Of the 32 TGF-β signaling molecules they detected changes in, 22 (including *Ctss, Ctsf*) were identified in MGM3, further suggesting this module is involved in TGF-β signaling and that TGF-β could be regulating the immune changes.

The perturbation in TGF-β signaling in MGM3 has many functional implications for the effects of alcohol on microglia. TGF-β signals to SMADs, and SMAD3 knockout mice have impaired immune function, suggesting SMAD signaling is involved in

regulating the immune response (X. Yang et al. 1999; Datto et al. 1999). Additionally, TGF-β and SMAD levels are altered following brain injury and neurodegeneration (Bernhardi et al. 2015; Villapol et al. 2013). Thus, the perturbation in TGF-β signaling we observed in microglia could be responsible for the altered immune profile. TGF-β can also regulate excitotoxicity (Boche et al. 2003), which is often a consequence of glial activation and thought to be a mediator of ethanol-induced neurodegeneration (Vetreno & Crews 2014).

TGF-β signaling is also responsible for controlling important cellular processes such as differentiation, maturation, and survival of CNS cells (Villapol et al. 2013). There is evidence that ethanol disrupts these processes as ethanol-induced TGF-β1 signaling promotes neuronal apoptosis during development (C. P. Chen et al. 2006). In addition, TGF-β2 can regulate synaptic transmission (Dobolyi et al. 2012), which indicates that altered TGF-β signaling may contribute to alterations in synaptic function in response to chronic ethanol. THM13, which was upregulated in response to ethanol, is enriched for biological processes related to synaptic plasticity. TGFβ is also important for survival of dopamine neurons (Roussa et al. 2009), which play an important role in the rewarding properties of ethanol. Therefore, altered TGFβ signaling could be altering dopamine transmission and contributing to the dependent phenotype.

Although TGF-β has been implicated in ethanol-induced liver injury (Dooley & Dijke 2012) and expression is increased in the blood of alcohol dependent subjects (Kim et al. 2009), no studies have investigated the role of TGF-β in ethanol-induced immune

signaling in the brain. Thus, future studies would benefit from investigating TGF-β signaling in the brain following ethanol consumption. Futhermore, although there haven't been any studies thus far investigating the interaction between TGFβ and Siglec-H, there is evidence for other Siglecs regulating TGFβ signaling in immune cells (Takamiya et al. 2013; Wu et al. 2016). Thus, the interaction between Siglec-H and TGFβ in microglia is also worthy of investigation.

4.V. Conclusions

The transcriptional landscape of microglia is a significant faction within the CNS immune system. Intermingled with other cell-types, microglia are instrumental in maintaining CNS homeostasis. The innate and adaptive immune systems are proposed as interrelated cellular mechanisms involved in chronic alcohol and excessive alcohol abuse (Vetreno & Crews 2014). CNS cell composition varies by brain region, influencing enduring immune responses and neurobehavioral traits. Identifying the molecular response of individual cell-types provides a foundation for inferring the immunomodulatory effects that are instigated.

Neuroimmune signaling can affect neuronal transmission and actions of alcohol (Bajo et al. 2014; Bajo, Herman, et al. 2015). Although gene expression alterations are not entirely equivalent with protein levels due to cellular stochastic processes increased coordinate gene expression within microglia following alcohol consumption is consistent

with the activation and proliferation of microglia in alcoholic brain tissue (Dennis et al. 2014). The associated gene expression puts forth a broader portrait of an alcohol-responsive microglial gene network, which is notably distinct from other CNS- and immune-related cell-types. Microglial studies in alcohol dependence and other psychiatric conditions have mainly focused on a role in neurotoxicity (M. A. Pascual et al. 2011; Alfonso-Loeches et al. 2010; Crews et al. 2006), but our studies indicate that voluntary alcohol consumption which is much less toxic has marked effects on the microglial transcriptome and suggest that microglia may regulate alcohol consumption. Several genes within this microglial network (*Ctss, Ctsf, Tlr3, Tlr9, Irak1, Casp8, and Ctsb*) have been implicated in AUDs, further validating this module (Blednov, Ponomarev, et al. 2011; Qin & Crews 2012; Lippai et al. 2013; Antón et al. 2016; Pla et al. 2016). The cathepsins, *Ctss* and *Ctsf,* have been causally implicated in alcohol consumption, suggesting that other members of this module may regulate consumption. These results also suggest the involvement of many additional microglial genes that have not previously been studied with alcohol.

Of particular importance in this study, was the identification of MGM3, which contains co-expressing microglial genes that are regulated by ethanol. Within this module, we highlighted TLR and immune signaling, possibly regulated by the hub gene *Siglech*. In addition, this module presented the novel hypothesis that chronic ethanol is regulating TGFβ signaling. The perturbations in TGFβ could be regulating the changes in immune signaling, as well as driving changes in synaptic plasticity (**Fig 4.5**). Further

studies are necessary to elucidate to the role of TGFβ and Siglec-H in modulating the response to ethanol.

The transcriptome of isolated cells is informative for clarifying target genes and functional networks that may be important for ameliorating imbalanced systems (Lamb et al. 2006). Brain pathologies exemplified by disturbances in CNS immune function benefit from surveying the transcriptome of isolated microglia. Animal models incorporating such cellular preparations refine gene expression signatures procured from an admixture of cell-types present within homogenized tissue, permitting an in-depth characterization of particular cellular responses. Discriminating the microglia-specific perturbations initiated can call attention to a series of subtle molecular modifiers of immune system function relevant to human conditions. Our results emphasize a cluster of microglial genes aligned in relation to alcohol consumption. Further research into microglial gene networks, and the development of molecular tools to selectively manipulate microglia, will uncover CNS immune responses inherent to substance abuse and dependence.

4.VI. Funding and Disclosure

The authors declare no conflicts of interest.

The authors would also like to gratefully acknowledge the support of the National Institutes of Alcohol Abuse and Alcoholism (NIAAA) through grants AA024654 (GMM), (add training grants SPF), AA013520 (YAB & RAH), and AA020926 (RDM & SPF).

4.VII. ACKNOWLEDGEMENTS

The authors would like to thank Jillian Benavidez, Courtney Bridges, and the Genomic Sequencing and Analysis Facility (University of Texas at Austin) for their technical assistance.

4.VIII. FIGURES

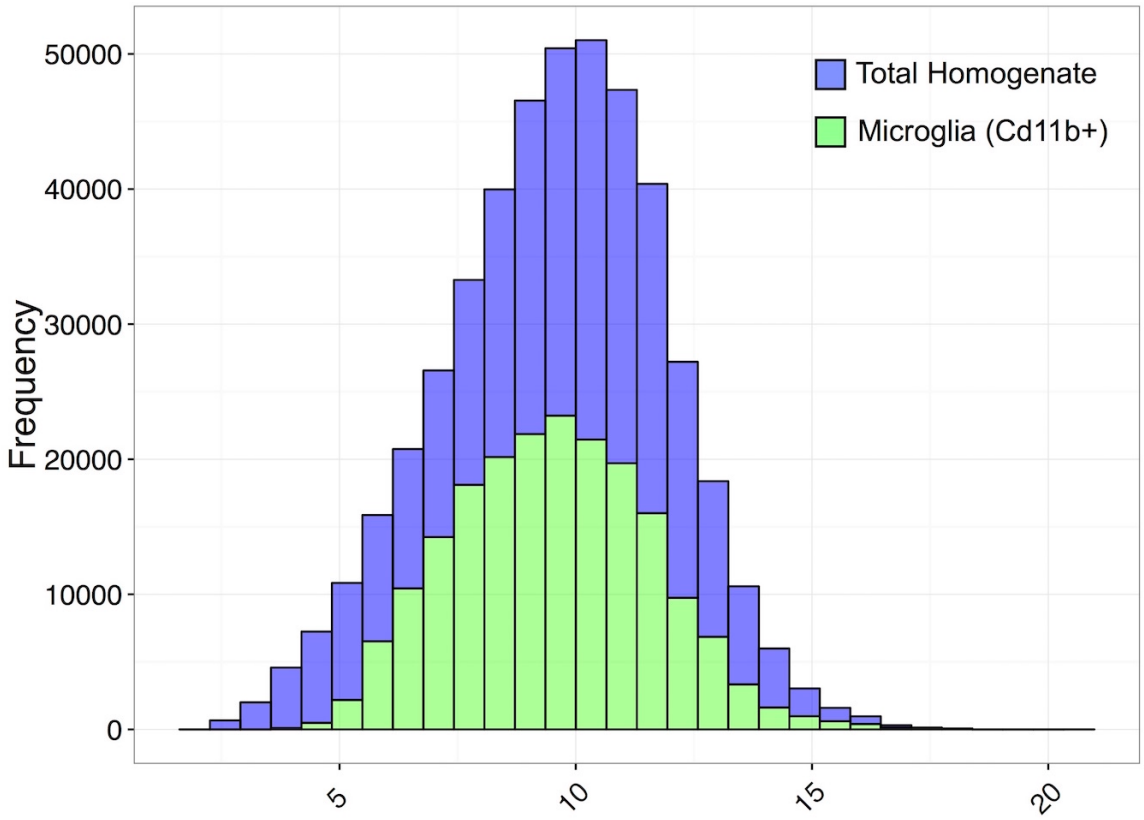


Figure 4.1: Read count distribution

Frequency distribution of $\log_2$ normalized counts for the total homogenate and microglia (Cd11b+). The total homogenate has a higher frequency of counts than the microglia.

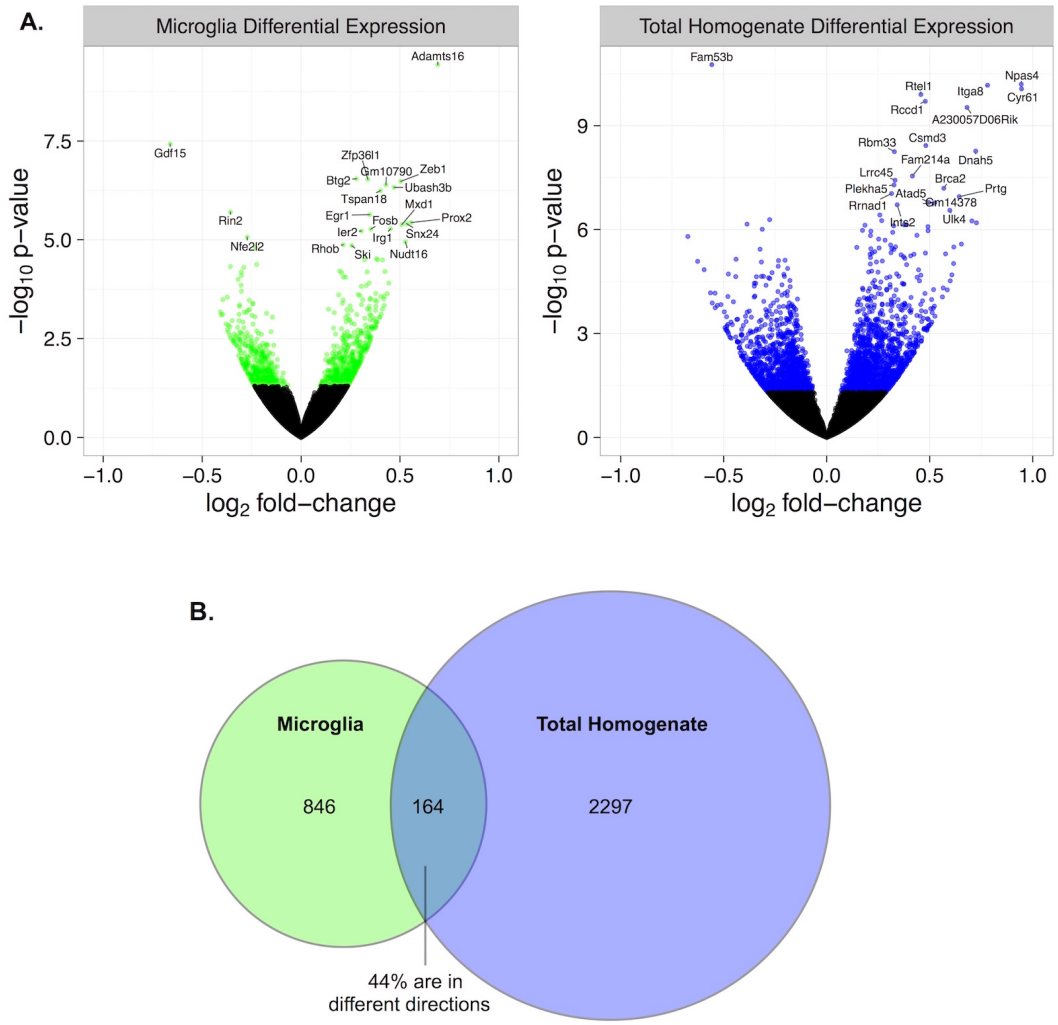


Figure 4.2: Differential Expression

Differential expression in response to chronic ethanol for microglia and total homogenate. A. Volcano plot showing $\log_2$ fold-change against $\log_{10}$ p-value. Differentially expressed genes ($p < .05$) are shown as green or blue dots. The top 20 differentially expressed genes are labeled. The total homogenate has larger fold-changes and lower p-values than the microglia. B. Venn Diagram showing the number of unique and overlapping differentially expressed genes ($p < .05$) in each of the preparations. 164 genes were differentially expressed with alcohol in both the microglia and total homogenate. Of these 164 genes, 44% (72 genes) were changed in different directions in the two preparations.

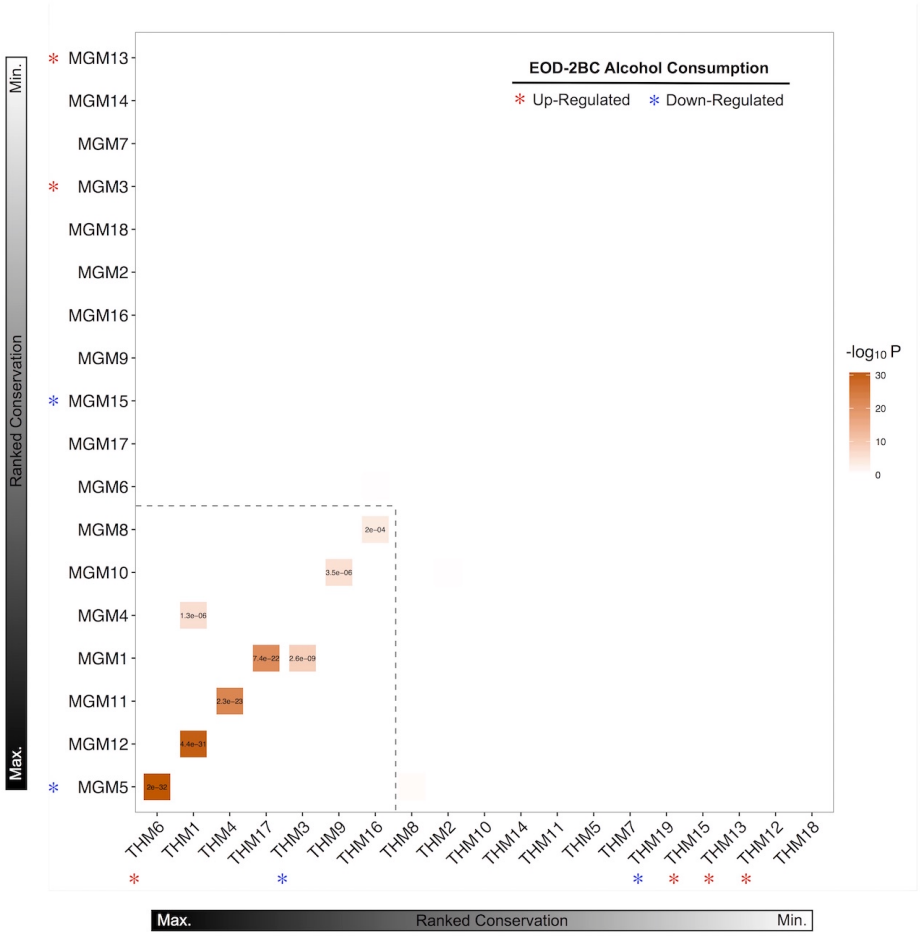


Figure 4.3: Microglial and Total homogenate module overlap

Overlap between the microglia and total homogenate modules based on gene membership (Fishers-exact-test, correct for multiple comparisons using a false discovery rate less than .05). The modules contained in the hatched box show high overlap between the two fractions and likely represent shared genes and processes. The modules outside the hatched box are unique to that fraction. * denotes differentially expressed with ethanol ($p < .05$) and the color indicates the direction of the change.

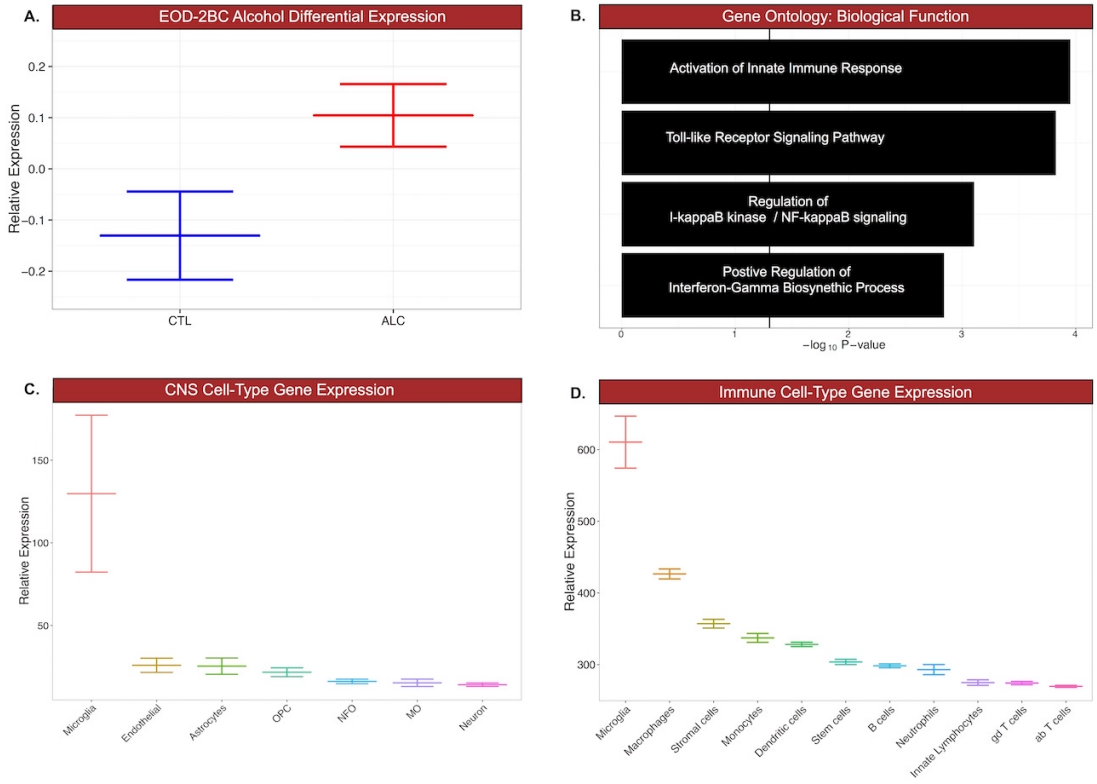
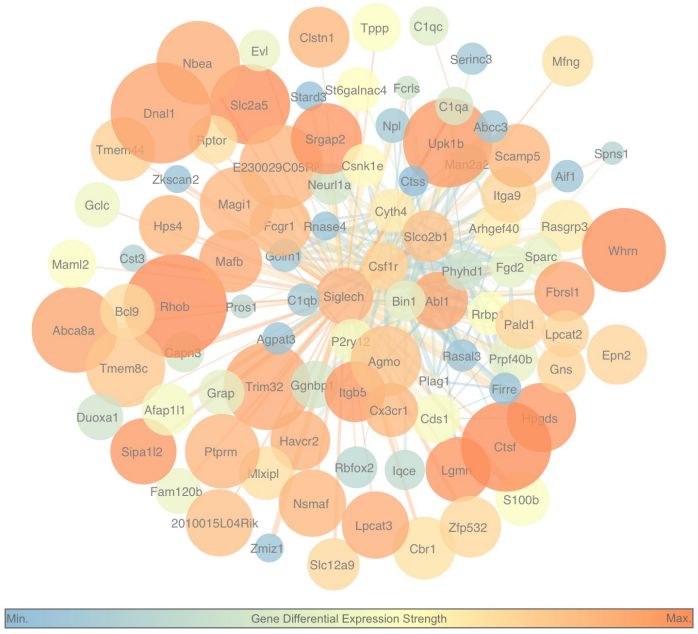


Figure 4.4: Microglial module 3

Microglial module 3 (MGM3) was selected for further investigation based on its differential expression, biological function, and microglial specificity. A. MGM3 shows increased expression with chronic ethanol consumption ($p < .05$, Kruskal-Wallis Rank Sum Test). B. Genes contained in MGM3 are associated with several GO Biological Processes related to innate immune signaling. C. When compared to other CNS cell types, MGM3 shows highest relative expression of microglial genes ($p < .05$, ANOVA). D. When compared to other immune cell types, MGM3 shows highest relative expression of microglial genes ($p < .05$, ANOVA).

A.



B.

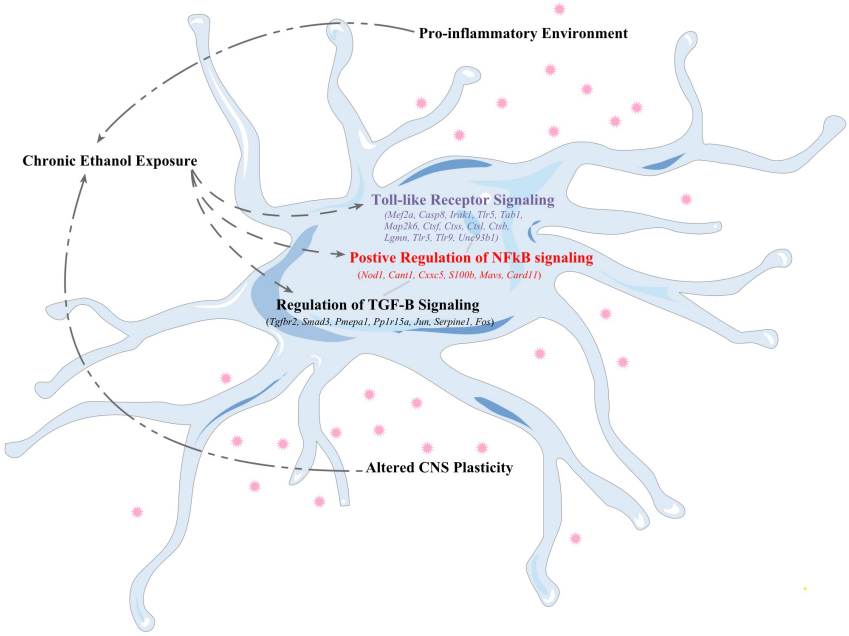


Figure 4.5: Summary of Microglial response

Figure 4.5: Continued

A. Connectivity map containing the top 250 connections in MGM3. Warmer colored nodes have smaller p-values for differential expression with alcohol. Larger nodes have larger fold changes with alcohol. Larger connecting lines indicate higher topological overlap measure (TOM), or connectivity between 2 genes. Warmer colored lines indicate higher edge betweenness centrality, or importance of the connection in the network. *Siglech* is highly connected to many genes in MGM3. B. Hypothesis about the effects of chronic ethanol on microglia in the frontal cortex. Chronic ethanol exposure leads to expression changes of genes involved in Toll-like receptor signaling, regulation of NF-κB, and TGF-β signaling. These gene expression changes lead to a pro-inflammatory environment and microglial dysfunction contributing to altered CNS plasticity. Cortical alterations in CNS plasticity then promote additional alcohol consumption.
Figure created using http://servier.com/Powerpoint-image-bank

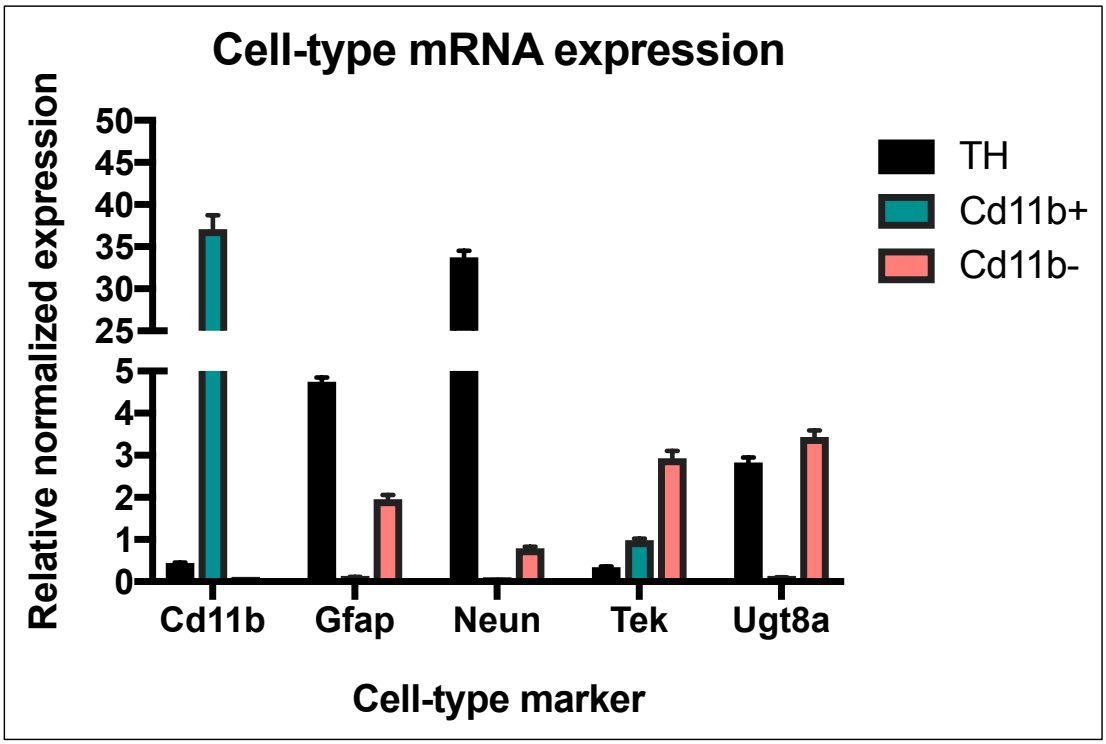


Figure 4.6: qPCR validation of isolation

Relative expression of cell-type markers in the total homogenate (TH), microglia (Cd11b+) and cells remaining after isolation (Cd11b-) measured using RT-qPCR. *Neun* was used as a neuronal marker, *Gfap* as an astrocyte marker, *Cd11b* as a microglial marker, *Tek* as an endothelial cell marker, and *Ugt8a* as an oligodendrocyte marker. The Cd11b+ fraction is highly enriched for *Cd11b* compared to other cell type markers. The Cd11b- fraction shows low levels of *Cd11b*, indicating that most of the microglia were successfully isolated in the *Cd11b*+ fraction.

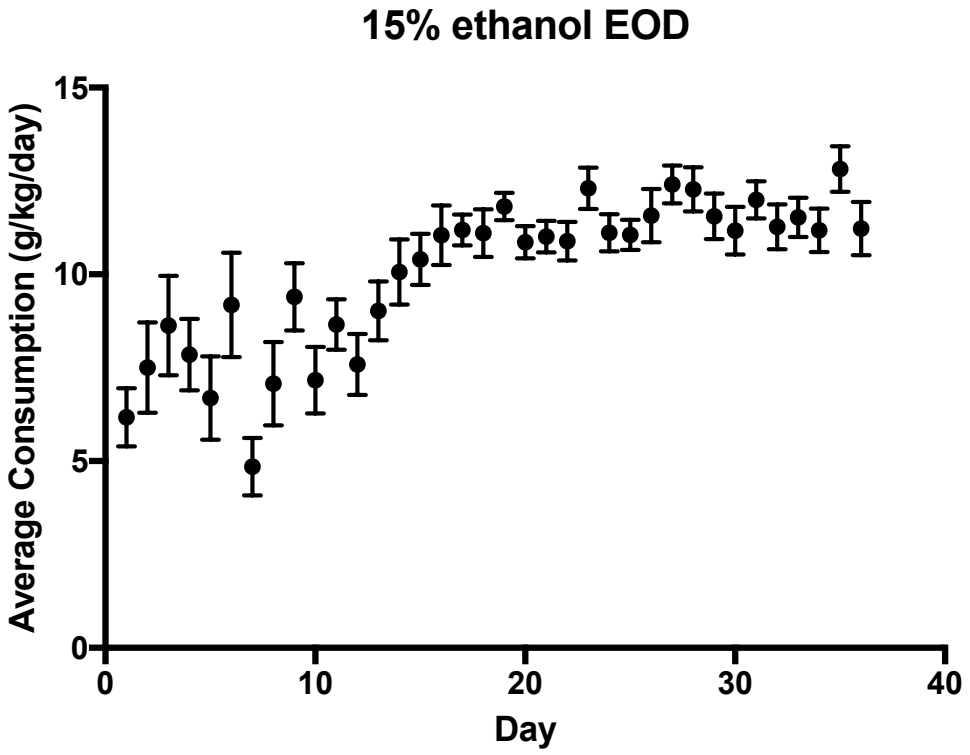


Figure 4.7: Ethanol consumption

Ethanol consumption data for every other day 2 bottle choice using 15% ethanol. Each data point is the average of consumption data for all 12 mice in a 24-hour period. Days represent only days where alcohol was present (every other day).

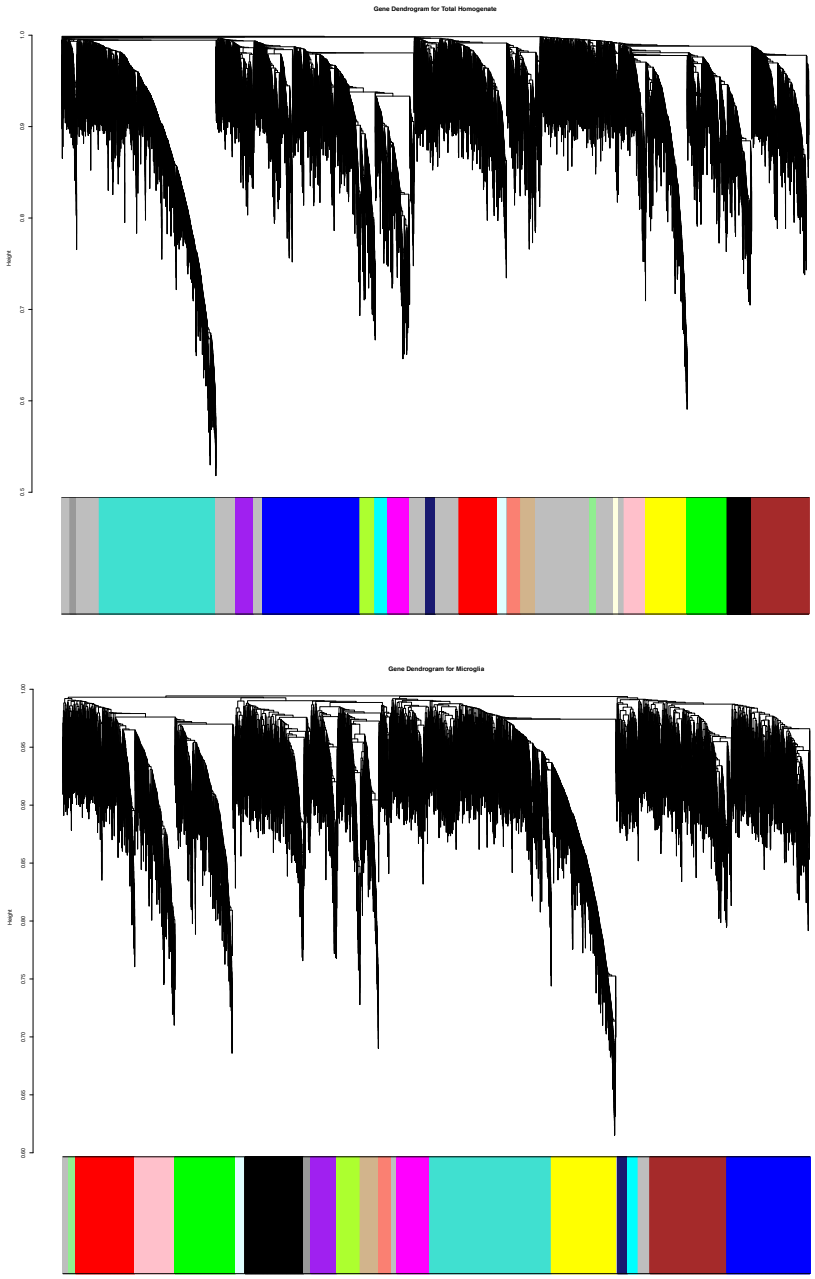


Figure 4.8: Gene dendograms

Gene dendograms for modules in the total homogenate and microglia.

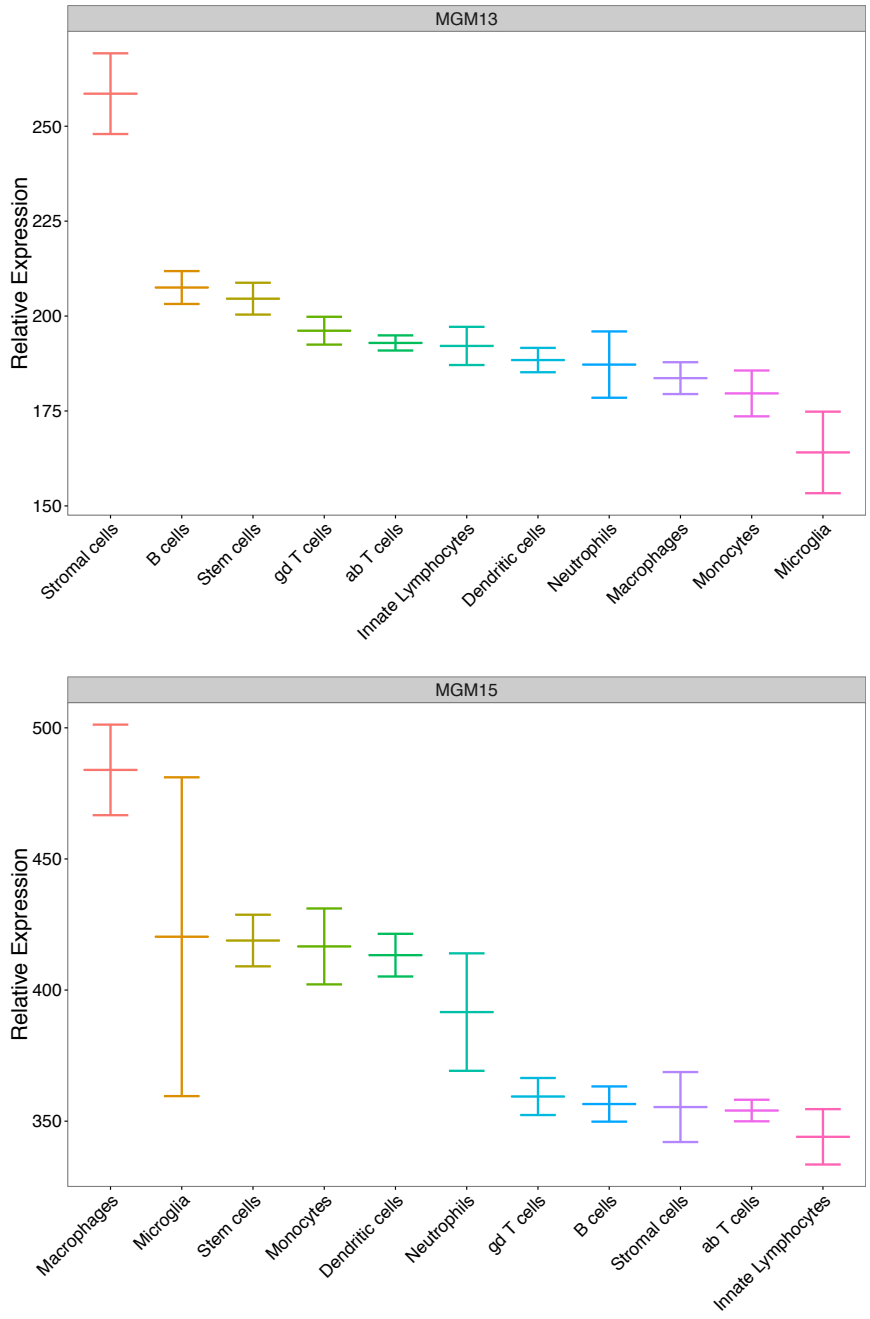


Figure 4.9: MGM13 and MGM15 cell-type enrichment

Figure 4.9: Continued

Comparison of the other two differentially expressed microglial modules, MGM13 and MGM15, to other immune cell types. A. MGM13 shows highest relative expression of stromal cell genes. B. MGM15 shows highest relative expression of macrophage genes. These two modules do not show the same microglial signature as MGM3 does.

4.IX. TABLES

Gene Symbol	Gene Name	Microglia Statistic	Microglia P-value
Tgfbr2	transforming growth factor, beta receptor II	2.32	2.01E-02
Ctsf	cathepsin F	2.67	7.69E-03
Smad3	SMAD family member 3	2.68	7.28E-03
Lgmn	legumain	2.07	3.83E-02
Serpine1	serine (or cysteine) peptidase inhibitor, clade E, member 1	2.25	2.43E-02
Zfp36l1	zinc finger protein 36, C3H type-like 1	5.13	2.90E-07
Rhob	ras homolog family member B	4.35	1.34E-05
Etv5	ets variant 5	3.59	3.25E-04
Apbb1ip	amyloid beta (A4) precursor protein-binding, family B, member 1 interacting protein	2.65	7.96E-03
Gpr56	adhesion G protein-coupled receptor G1	4.01	6.19E-05
Ccl12	chemokine (C-C motif) ligand 12	2.54	1.10E-02
Cd276	CD276 antigen	3.36	7.93E-04
Emr1	adhesion G protein-coupled receptor E1	2.09	3.69E-02
Rnf135	ring finger protein 135	2.81	5.00E-03
Tnfsf12Tnfsr13	tumor necrosis factor (ligand) superfamily, membrane-bound member 13	2.08	3.75E-02
Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha	2.92	3.49E-03
Il10ra	interleukin 10 receptor, alpha	2.55	9.00E-04
Klf4	Kruppel-like factor 4 (gut)	3.79	1.50E-04
Rtn4rl1	reticulon 4 receptor-like 1	3.39	6.98E-04
Numb	numb homolog (Drosophila)	2.47	1.36E-02

Table 4.1: Differentially expressed microglial genes in MGM3

Table of some MGM3 genes that are differentially expressed in microglia but not in the total homogenate. Gene symbol, gene name, T-statistic and p-value are given. All genes in the table show no significant change in the total homogenate.

Chapter 5: Microglial gene expression changes 1-week after LPS and comparison to ethanol

Gizelle M. McCarthy, Yuri A. Blednov, R. Adron Harris, and R. Dayne Mayfield

5.I. Abstract

Alcohol Use Disorder (AUD) is a widespread and debilitating disorder that is characterized by the inability to stop consuming alcohol. Altered immune signaling in the prefrontal cortex (PFC) is one proposed mechanism by which chronic alcohol use changes behavioral control. The neuroimmune changes that occur in response to alcohol are often compared to the changes induced by the endotoxin lipopolysaccharide (LPS). Furthermore, ethanol-induced LPS leakage into the blood stream is thought to mediate the neuroimmune response. Although previous studies have identified the gene expression changes that occur in the brain following LPS exposure, none have characterized the changes that are occurring specifically in microglia, the immune cell of the brain. In this study, we profiled the gene expression changes that occur in PFC microglia 1 week after peripheral LPS administration. In addition, we compared the gene expression changes from LPS to ethanol, and found that although post treatments altered immune signaling in microglia, they changed different specific processes and pathways. Comparison to previous data sets suggest that the overlap between ethanol and LPS gene expression changes might be attributed to other non-microglial cell types. Together these data reveal that the microglial response in the PFC differs between ethanol and LPS, suggesting that distinct mechanisms are mediating the neuroimmune response to these two treatments.

5.II. INTRODUCTION

Alcohol Use Disorders (AUDs) are major problem in United States, with a lifetime prevalence of 29.1% (Grant et al. 2015). AUDs are highly comorbid with other psychiatric disorders and lead to disability for those effected and an economic burden for the country. Despite the prevalence, only 20% of individuals with AUDs ever seek treatment, emphasizing the need for new and better therapeutic options. Dysregulated immune signaling in the brain has recently emerged as a mediator of alcohol dependence, and due to the number of existing immunomodulatory drugs, has potential to be a therapeutic target. Thus, it is important to understand how immune signaling occurs in response to alcohol and how this differs from other well characterized immune responses.

The innate immune response to the bacterial endotoxin lipopolysaccharide (LPS) has been well characterized, and the immune response to ethanol has frequently been compared to LPS (Alfonso-Loeches et al. 2010; M. A. Pascual et al. 2011; Bajo et al. 2014; Fernandez-Lizarbe et al. 2009). Peripheral LPS administration leads to increased immune signaling in the brain, although it is hypothesized to be an indirect response (Bossù et al. 2012). Chronic alcohol use alters the intestinal microbiome and compromises the tight junctions in the intestines, resulting in increased permeability and bacterial 'leakage' into the blood stream (Ferrier et al. 2006; Keshavarzian et al. 1999). Consistent with this idea, increased intestinal permeability and serum endotoxin levels are seen in alcohol dependent subjects (Leclercq et al. 2012; Leclercq, Matamoros, et al. 2014). Increased circulating endotoxin leads to activation of the LPS ligand Toll-like receptor 4 (TLR4) and production of proinflammatory cytokines (Leclercq, De Saeger, et

al. 2014). Pro-inflammatory cytokines in the blood stream can then lead to immune activation and amplification in the liver and other peripheral tissues. Although LPS is a large molecule that does not cross the healthy blood-brain barrier, cytokines (e.g. TNFα) that are increased in response to peripheral LPS do (Szabo & Lippai 2014; Bossù et al. 2012; Lippai et al. 2013). CNS inflammation causes neurodegeneration and increased ethanol consumption in rodents, leading to the hypothesis that immune signaling in the brain contributes to alcohol induced brain damage and dependence (Qin et al. 2007; Blednov, Benavidez, et al. 2011).

Microglia are the immune cells of the brain and are thought to mediate at least a part of the neuroimmune response to alcohol and LPS. Studies using cultured primary microglia have demonstrated that both ethanol and LPS lead to microglial activation and TLR4 signaling *in vitro* (Fernandez-Lizarbe et al. 2009; Fernandez-Lizarbe et al. 2013). However, it is becoming clear that although LPS and ethanol both activate TLR signaling, their effects on the immune response in the CNS differ *in vivo* (Whitman et al. 2013). The differences in effects are not suprising considering the distinctions between LPS and ethanol. For example, ethanol can cross the blood-brain barrier while LPS remains in the periphery and is thought to activate the CNS immune response through peripheral cytokine release (Banks & S. M. Robinson 2010; Qin et al. 2007). LPS is also a direct ligand for TLR4, while ethanol is believed to activate TLR signaling through increases in endogenous TLR ligands (Whitman et al. 2013). In addition, ethanol has multiple effects on distinct cell types within the brain; thus, it is likely that other effects of ethanol modulate immune signaling. Additional work is needed to better understand

the similarities and distinctions between the effects of alcohol and LPS on microglia *in vivo*.

A previous study compared gene expression profiles in the prefrontal cortex (PFC) following various chronic ethanol exposures and LPS administration (Osterndorff-Kahanek et al. 2013). These data showed that every other day ethanol consumption and LPS (1 week after administration) had a significant number of overlapping gene expression changes, most of which changed in the same direction. Although these results suggested an overlap in the immune response, it is unclear how the microglial-specific changes are similar and different between the two treatments. Recent advances in microglia isolation techniques from brain have allowed for closer evaluation of cell-type specific changes in the adult CNS. We sought to define the microglial response to peripheral LPS using isolated microglia from mouse PFC. Furthermore, we compared the microglial LPS response to our recent data profiling the microglial ethanol response (chapter 4). Our results highlight some unexpected microglial changes in response to LPS and highlight distinct microglial response to LPS and chronic ethanol.

5.III. Materials and Methods

5.III.a. Ethics statement

All procedures were approved by the University of Texas at Austin Institutional Animal Care and Use Committee (animal protocol number AUP-2013-00061) and adhered

to the NIH Guidelines. The University of Texas at Austin animal facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

5.III.b. Animals and LPS administration

Studies were conducted in adult (6-8 weeks old) C57Bl/6J male mice (Jackson Laboratories, Bar Harbor, ME). Mice were individually housed and allowed to acclimate to upright bottles one week before the start of the experiment. The experimental rooms were maintained at an ambient temperature of 21±1°C, 40-60% humidity, and a regular light/dark schedule (7 AM-7 PM). Food and water were available *ad libitum*. Mice were randomly assigned to either LPS or saline groups (n=5/group). Mice were weighed one day prior to LPS (1.5 mg/kg) or saline injection (intraperitoneally; IP). Mice were weighed daily and sacrificed after 1 week (**Fig. 5.4**). The one week time point was chosen because previous data has shown that ethanol consumption is increased 1 week after acute LPS and that PFC gene expression changes at 1 week overlapped with every other day ethanol consumption (Blednov, Benavidez, et al. 2011; Osterndorff-Kahanek et al. 2013).

5.III.c. Microglia and RNA Isolation

Mice were perfused with ice-cold saline, brains were removed, and the PFC was dissected as described previously (Osterndorff-Kahanek et al. 2013). The tissue was minced on ice, re-suspended in cold HBSS followed by microglial isolation as described by Nikodemova et al. 2015. Briefly, tissue suspension was enzymatically dissociated

using the Neural Tissue Dissociation Kit- Papain (Miltenyi Biotec, Germany) in conjunction with Pasteur pipette manual dissociation. Dissociated tissue was passed through a 70 μM strainer (Miltenyi Biotec), centrifuged at 300 x g, then resuspended in 30% Percoll (Sigma-Aldrich, St. Louis, MO). The Percoll-cell suspension was centrifuged at 700 x g for 15 minutes at room temperature to separate the myelin from the isolated cells, and the myelin was subsequently removed. Cells were washed and then incubated with CD11b MicroBeads (Miltenyi Biotec) and eluted using MS columns to collect CD11b+ cells. CD11b+ cell pellets were collected by centrifugation at 300 x g for 10 minutes at 4°C and then the supernatant was removed and the pellet flash frozen. RNA was isolated using the RNeasy Micro Kit (Qiagen, Germany). RNA concentration and quality (RNA integrity number) was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) with the Agilent RNA 6000 Pico Kit.

5.III.d. RNA Sequencing and Analysis

Samples were Poly(A) selected using the Poly(A) Purist Kit (Thermo Fisher Scientific Inc.) and prepared using NEBNext® Ultra™ Directional RNA Library Preparation Kit (New England BioLabs, Ipswich, MA). Samples were sequenced on the NextSeq 500 (Illumina, San Diego, CA) using (75 bp; paired-end reads) to a minimum target depth of 20 million reads per sample. Reads were mapped to the mouse reference genome (UCSC, mm10) using the alignment tool STAR (Dobin et al. 2013). Raw RNA-sequencing files were evaluated for quality control using FastQC (0.11.13). HTSeq, was

used for counting mapped sequencing reads. Quantified read counts were normalized and analyzed for differential expression using DESeq2 (v 1.10.1), between saline and LPS treated isolated microglia, using the R statistical computing environment (v3.3.1)(Love et al. 2014). Statistical significance was assessed using a nominal p-value less than or equal to 0.05. Individual gene sets were functionally annotated using the web-based software application Enrichr (Kuleshov et al. 2016; E. Y. Chen et al. 2013)

5.III.e. Comparison to other data sets

The microglial response to LPS was compared to the microglial response to chronic voluntary ethanol consumption using previously published data (chapter 4). Additional analyses compared the microglial LPS and ethanol overlap to previously published microarray data that profiled the PFC including all cell types in response to 1 week post-LPS treatment (Osterndorff-Kahanek et al. 2015).

5.IV. Results and Discussion

5.IV.a. Microglial transcriptome 1 week after LPS

RNA sequencing was used to evaluate gene expression changes in the PFC one week after IP LPS administration. A total of 17,192 genes were detected (genes were excluded that had no reads detected in any sample), and differential expression analysis identified 898 differentially expressed genes at a p value less than .05. The p value cut off was based on a previous study that measured alcohol and 1 week post-LPS gene

expression differences (Osterndorff-Kahanek et al. 2013)(*Chapter 4: Microglia-specific transcriptome changes following chronic alcohol consumption*). Of the 17,192 genes detected in this study, 470 genes were upregulated and 428 were downregulated. Enrichment analysis revealed that the most significantly enriched biological processes were driven by differential expression of ribosomal RNAs. These biological processes included RNA catabolic process (GO:0006401), protein targeting to ER (GO:004507) and translation (GO:0006412) (**Fig. 5.1**). The enrichment of ribosomal RNAs was not expected given that LPS treatment results in immune signaling activation and ribosomal genes have been used as housekeeping genes in LPS studies (van Schaarenburg et al. 2016; Siegfried et al. 2013). However, given the importance of altered gene expression in immune signaling, it expected that innate immune signaling would change regulation of transcription, RNA processing, and translation (Carpenter et al. 2014). GWAS studies have identified ribosomal protein L5 (*Rpl5*) as a susceptibility gene for Multiple Sclerosis (Rubio et al. 2008) and *Rpl13* as a susceptibility gene for Alzheimer's Disease (De Jager et al. 2014). Both of these CNS disorders involve microglial activation and recent studies suggest that increased expression of ribosomal proteins in activated microglia may play an important role in the inflammatory response in the CNS (Lee et al. 2012). Furthermore, gene expression analysis of microglia isolated from a mouse model of neurodegeneration(Vincenti et al. 2016) and aged mice (Orre et al. 2014) revealed several differentially expressed genes related to translation that were also differentially expressed in our LPS data (e.g. *Rpl5, Rpl12, Eif4b)*. Therefore, ribosomal RNAs represent a potential area that deserves more study with regards to microglial activation, and careful

consideration should be taken before using these genes as endogenous controls in inflammatory models.

There were also several gene expression changes related to the response to topologically incorrect protein (GO:00035966), including many genes encoding heat shock proteins. LPS treatment in macrophages induces ER stress, splicing, and unfolded protein response (UPR) that amplifies immune signaling through cytokine production (Grootjans et al. 2016). Furthermore, misfolded proteins are able to activate microglia via TLRs, and decreased phagocytosis of unfolded proteins in response to immune stimuli is thought to contribute to the pathology of neurodegenerative diseases (Heneka et al. 2014).

As expected, the inflammatory response (FO:0006954) was altered 1 week after LPS, including increases in several cytokines (e.g. *Tnf, Il1b, Ccl5, and Cxcl10*). These results are consistent with other studies that have showed long lasting cytokine responses to a single LPS exposure (Bossù et al. 2012). TNFα signals through two different receptors and regulates many cellular processes, including cytokine production, cell adhesion, gliosis, cell survival and proliferation, and apoptosis (Kraft et al. 2009). Consequently, the differentially expressed biological processes also include positive regulation of tumor necrosis factor superfamily cytokine production (GO:1903555), regulation of cell motility (GO:2000147), and regulation of apoptotic signaling pathway (GO:2001233) (**Fig. 5.1**). In addition, TNFα signaling impacts other cells within the CNS. For example, microglial TNFα can potentiate glutamate toxicity by binding to

astrocytes and neurons (Olmos & Lladó 2014), can cause demyelination through its effects on oligodendrocytes (Probert 2015), and can induce apoptosis in neuronal progenitor cells (Guadagno et al. 2013).

5.IV.b. Microglial response to LPS differs from total homogenate response

Previous work used microarrays to evaluate PFC gene expression changes in the total homogenate 1 week after LPS and found that 1049 genes were differentially expressed (Osterndorff-Kahanek et al. 2013). In contrast to the microglial genes, these genes were enriched for biological processes related to positive regulation of protein kinase activity (GO:0045860). Comparison between the total homogenate and microglia differential expression data revealed an overlap of 77 genes. Like the microglial ontologies, many of the overlapping genes were related to RNA catabolic process (GO:0006401) and regulation of translation (GO:0006417). These results suggest that while microglia have many changes related to regulation of mRNA and protein expression, changes in these processes can also be detected in the total homogenate 1 week after LPS.

5.IV.c. Comparison of microglial response to LPS and ethanol

In a recent study, we used isolated microglia to profile the microglial response to chronic ethanol consumption (Chapter 4). Immune changes resulting from ethanol administration are often compared to the immune changes induced by LPS, so we were interested in investigating how microglial gene expression changes differed between the two treatments. A comparison of differential expression data revealed that LPS (1-week

post-administration) had greater fold-changes than changes observed in response to chronic ethanol administration, although all were less than 2-fold (**Fig. 5.2**). There were a great number of differentially expressed genes with p values < .05 in the ethanol group compared to the LPS group; however, this is likely due to the smaller sample size in the LPS group.

Evaluation of differential expression within each treatment revealed several overlapping biological processes, although many expression changes were in different directions (**Fig. 5.2B**). Both groups showed increased expression of genes related to cell chemotaxis (GO:0060326), leukocyte migration (GO:0050900), and inflammatory response (GO:0006954). The genes driving these ontologies include chemokines and chemokine receptors (e.g. *Cxcr2, Ccl5*), metalloproteinases (e.g. *Adam8, Mmp9*), solute carriers (*Slc7a5, Slc7a8*), and integrins (*Itgal, Itgb2*). Metalloproteinases, particularly *Mmp9*, have recently emerged as important mediators of immune response, synaptic plasticity, cognitive impairments, and alcohol-seeking behavior (Stefaniuk et al. 2017; Y. Yang et al. 2011; Lasek 2016). Microglial migration, chemotaxis, and invasion are an important part of the neuroimmune response (Vincent et al. 2012). Previous studies have suggested that LPS alters microglial migration, although the direction of change varies (Lively & Schlichter 2013; Hu et al. 2014). There are few studies directly investigating microglial migration in response to ethanol, but there is evidence that ethanol alters chemotaxis in astrocytes (Davis & Syapin 2004) and liver macrophages (Kupffer cells)(Bautista 2002). Furthermore, several molecules involved in chemotaxis are

changed in the brain after chronic ethanol and regulate ethanol consumption (BLEDNOV et al. 2005; He & Crews 2008; Kane et al. 2013). Therefore, microglial migration is a potential process changed both with LPS and ethanol, and warrants further investigation in alcohol studies.

Although both treatments led to an increase in genes related to inflammatory response, they showed differences in specific inflammatory pathways. The LPS group showed increased expression of genes related to lipopolysaccharide-mediated signaling pathway (GO:0031663) and positive regulation of MAPK cascade (GO:0043410), while decreased expression was observed in the ethanol group. Thus, it appears that while LPS produces a typical pro-inflammatory response even a week after administration, ethanol does not. Microglial MAPK signaling is activated by TNFα, and is responsible for LPS-induced neuronal damage (Xing et al. 2011). Given that TNFα expression was upregulated in the LPS-treat group, but downregulated in the ethanol-treated group, MAPK signaling might also be expected to change in opposite directions. Furthermore, microglial MAPK signaling promotes apoptosis{Xie:2010in(Xie et al. 2010), and several genes related to apoptosis were also increased with LPS and decreased with ethanol. Previous studies have demonstrated increased microglial MAPK expression following ethanol exposure *in vitro*, however, this was accompanied by an increase in TNFα expression (Fernandez-Lizarbe et al. 2009). Therefore, it appears that microglial cytokine and MAPK expression is regulated differently in response to chronic ethanol *in vivo*. Because MAPK signaling can regulate many processes, including growth, differentiation,

apoptosis, and inflammation, it is an attractive target for modulating the neuroimmune response.

In contrast, genes that were upregulated with ethanol and downregulated with LPS were enriched for transforming growth factor beta signaling pathway (0007179) and response to topologically incorrect protein (GO:0025966). TGF-β signaling has recently emerged as an important component regulator of microglial function and immune response, and loss of TGF-β in the CNS results in reduced microglial numbers, impaired synaptic plasticity, and deficits in extracellular glutamate (Butovsky et al. 2013; Koeglsperger, Li, Brenneis, Saulnier, Mayo, Carrier, Selkoe & Weiner 2013a). Studies suggest that increased TGF-β signaling inhibits expression of chemokines and receptors, genes responsible for cell migration, apoptosis, and infection response (Paglinawan et al. 2003). Some of these genes inhibited by TGF-β (*Ccl3, Samhd1, Vcam1, Tnf, Gbp2)* were found to be upregulated with LPS and downregulated with ethanol. However, there are some of these inhibited genes that are upregulated with ethanol, suggesting that the response in vivo is more complicated and cytokine and TGF-β related signaling can increase simultaneously. In addition, TGF-β signaling involves cross-talk with multiple pathways, including MAPK, PI3K/Akt, and TNFα/IL/NF-κB (X. Guo & X.-F. Wang 2009). Nevertheless, TGF-β's interactions with many pathways makes it an interesting target in CNS disorders that involve neuroimmune dysregulation. In support of this, microglial TGF-β expression is increased and signaling is dysregulated in models of aging and neurodegeneration (Bernhardi et al. 2015). Although TGF-β is known to be

involved in the liver immune response to chronic ethanol, the influence of TGF-β signaling in the CNS response to ethanol has been unexplored and is worthy of future investigation.

Both treatments also had biological processes that were downregulated, including mitotic cell cycle (GO:0000278) (**Fig. 5.2**). Although down regulation of cellular processes may occur during inflammation, proliferating microglia usually have increased expression of cell-cycle genes. Cell cycle inhibition reduces the inflammatory response and provides neuroprotection, and could therefore be a compensatory mechanism in response to inflammation (Skovira et al. 2016). In keeping with this idea, TGF-β activation is known to cause cell cycle arrest (Hocevar & Howe 1998) and this response is seen in microglial cell lines in response to LPS (Kaneko et al. 2015) and in splenic NK cells isolated from alcohol consuming mice (Gallucci & Meadows 1996). The notch signaling pathway (GO:0007219) is also downregulated with both treatments and regulates immune response in microglia (Yao et al. 2013) and cell-cycle in other glial cells (Conner et al. 2014; Bongarzone et al. 2000). Therefore, downregulation of notch signaling and cell-cycle genes may serve to limit microglial proliferation in response to inflammation.

It is worth noting that although genes changing in one direction might be enriched for a pathway (e.g. TGF-β signaling, MAPK signaling, apoptosis), these pathways often include genes that both activate and inhibit signaling.

5.IV.d. Ethanol and LPS gene expression overlap

A comparison of the differentially expressed genes between ethanol and LPS revealed an overlap of 120 genes, which is greater than expected by chance (p=2.2e-16, Fisher's test) (**Fig. 5.3**). Of these 120 genes, 73% had expression changes in opposite directions, suggesting that even the overlapping genes are showing opposite responses (**Fig. 5.3A-B**). Investigation into the overlapping genes revealed that the ontologies for changes in the same and opposite directions were like those seen in all the DE genes (**Fig. 5.2**). The top ontologies for genes that changed in the same direction for both groups were extracellular matrix organization (GO:0030198) and integrin cell surface interactions (R-HAS-216083) (**Table 5.1**). The extracellular matrix (ECM) plays an important role in cell adhesion, synapse formation, regulation of neurotransmitter diffusion, as well as maintenance of the blood-brain barrier (Y. Yang et al. 2011; Lasek 2016). Cytokine expression and microglial activation are known to influence the expression of ECM genes, such as metalloproteinases and integrins (Milner & Campbell 2003). Furthermore, changes in ECM components can degrade the blood-brain barrier and propagate the immune response by acting as pathogen receptor ligands (Gaudet & Popovich 2014; Colton 2009). ECM components are known to change with ethanol administration and may regulate consumption (Lasek 2016), making microglial expression of these genes an interesting focus in future studies.

Genes that were upregulated with LPS and downregulated with ethanol were enriched for positive regulation of inflammatory response (GO:0050729) and consisted of several cytokines (*Ccl3, Ccl12, Tnfa, Il17ra)*. In contrast, genes that were upregulated

with alcohol but downregulated with LPS were related to regulation of transcription from RNA polymerase II promoter in response to stress (GO:0043618) and included several genes related to TGF-β signaling (*Smad3, Klf6, Pmepa1, Ubb)*. These changes are consistent with the with the cytokine/TGF-β changes seen looking at all the differentially expressed genes in each treatment.

5.IV.e. Comparison of microglial LPS-Ethanol overlap to total homogenate

In contrast to the differences in microglial response to LPS and ethanol, previous microarray data showed a high overlap between LPS and ethanol differentially expressed genes (Osterndorff-Kahanek et al. 2013). This study utilized RNA from the PFC (containing all cell types, referred to as total homogenate) and found that there was a greater overlap between LPS and chronic intermittent ethanol than expected by chance and that most of these changes were in the same direction. Given that most of these changes seemed to be unidirectional and that the majority of of the microglial changes were in the opposite direction, we were interested how the gene ontologies between the two differed (*Table 5.1: Ontologies for ethanol-LPS gene overlap in microglia and total homogenate*).

In contrast to the cytokine and TGF-β-related genes that distinguished LPS and ethanol in microglia, the overlap in the total homogenate was related to synaptic transmission (GO:0007268). Genes that were changed in the same direction were related to histone H3 deacetylation (GO:0007268) and neurotransmitter receptor binding and

downstream transmission in the postsynaptic cell (RHSA_112314). However, it is worth noting that many of the ontologies for these genes were driven by only a few genes, emphasizing the heterogeneity of the overlap. These results suggest that the common changes between LPS and ethanol may be more related to neuronal response than to microglial response. There are several studies that suggest LPS acutely alters synaptic transmission (Gao et al. 2014; Bajo, Varodayan, et al. 2015) and has long-term effects on spine dynamics (Kondo et al. 2011), behavior (Bossù et al. 2012; Valero et al. 2014) and cortical neuromodulation (Ming et al. 2015). Additionally, decreases basal firing of ventral tegmental area (VTA) dopamine neurons 7-10 days after LPS administration, confirming that synaptic activity remains changed at the 1 week time point (Blednov, Benavidez, et al. 2011). Although the mechanism of LPS changing synaptic transmission is likely an indirect effect through microglia (J. George et al. 2016; J. Zhang et al. 2014), there is work supporting direct effects of LPS on neurons (Leow-Dyke et al. 2012; Juan Liu et al. 2011). In contrast to LPS, ethanol is known to have direct effects on synaptic activity (Moriguchi et al. 2007), however, the overlap with LPS suggests a common mechanism as well, possibly through immune activation.

Histone deacetylation represses transcription and increased deacetylation is thought to counteract the inflammatory response (Yoza et al. 2002), as well as contribute to psychiatric disorders including alcohol use disorder (Sakharkar et al. 2014) . In neurodegenerative disease models histone deacetylase (HDAC) inhibitors improve degeneration, learning and memory, and synaptic plasticity (Konsoula & Barile 2012),

suggesting that these cognitive processes may mediate the effects HDAC inhibitors on alcohol consumption.

Although not the top ontology, several of the overlapping total homogenate genes were related to growth factor signaling, particularly FGF signaling. FGF signaling has important roles in neurogenesis, neuroinflammation, and psychiatric illness (Galvez-Contreras et al. 2016). FGF signaling influences and is influenced by TGF-β signaling, highlighting the role of growth factor signaling in the regulation of the neuroimmune response and alcohol use disorders.

A caveat of this comparison of LPS-ethanol overlap in total homogenate and microglia is that there were differences in experimental design. The microglial gene expression data were evaluated by RNA sequencing and analyzed using DESeq2, while the total homogenate gene expression was measured using microarray technology and analyzed using Limma. In addition, the total homogenate data was taken from female mice, and there are observed ethanol and immune differences between sexes (Alfonso-Loeches et al. 2013; Agrawal et al. 2013). Furthermore, the total homogenate ethanol mice were collected at a different time point (24 hours after ethanol removal), which could also contribute to differences.

5.V. Conclusions

Because the LPS response is well characterized and directly activates TLR signaling, other CNS disorders that activate immune signaling, like chronic alcohol use,

are often compared to LPS. However, the results presented in this chapter suggest that although LPS and ethanol both elicit an immune response, this microglial response differs between the two treatments. These results are consistent with a recent study that profiled spinal cord microglia from an Amyotrophic Lateral Sclerosis (ALS) mouse model and LPS treatment (Chiu et al. 2013). Despite previous comparisons between microglia in ALS and the microglial response to LPS, they found a distinct microglial ALS profile. Furthermore, they found that ALS microglia, like ethanol microglia, co-express typical anti-inflammatory and pro-inflammatory genes. These differences highlight the heterogeneity of the microglial response to disease and emphasize the need to characterize microglia by their stimulus *in vivo*, instead of trying to divide them into M1 (LPS-induced) or M2 (IL-10/TGF-β induced) categories (Ransohoff 2016a).

It is important to note that time is always a factor when evaluating neuroimmune response. Microglia in this study were taken immediately after ethanol removal, however, some studies suggest that the immune response to ethanol is biphasic and that largest immune changes in the brain are found 24-hours after ethanol removal (Whitman et al. 2013). Therefore, although ethanol and LPS response show little overlap in this study, they might show higher overlap at another time point. Furthermore, the idea of biphasic responses as well as ontologies including activators and inhibitors, makes direction of changes less important, and dysregulation of processes more important. Thus, microglia do show dysregulation in similar pathways in response to LPS and ethanol.

In summary, although LPS is known to activate microglia, this is the first study to characterize microglial changes 1 week after LPS exposure and these data provide new

insights into the more persistent microglial changes in response to pathogens. In addition, this study highlights the similarities and differences between the effects of alcohol and LPS on microglia and highlights new processes that may contribute to the microglial response.

5.VI. ACKNOWLEDGEMENTS

The authors thank Jillian Benavidez, Mendy Black, and Adriana DaCosta for their technical assistance.

5.VII. FIGURES

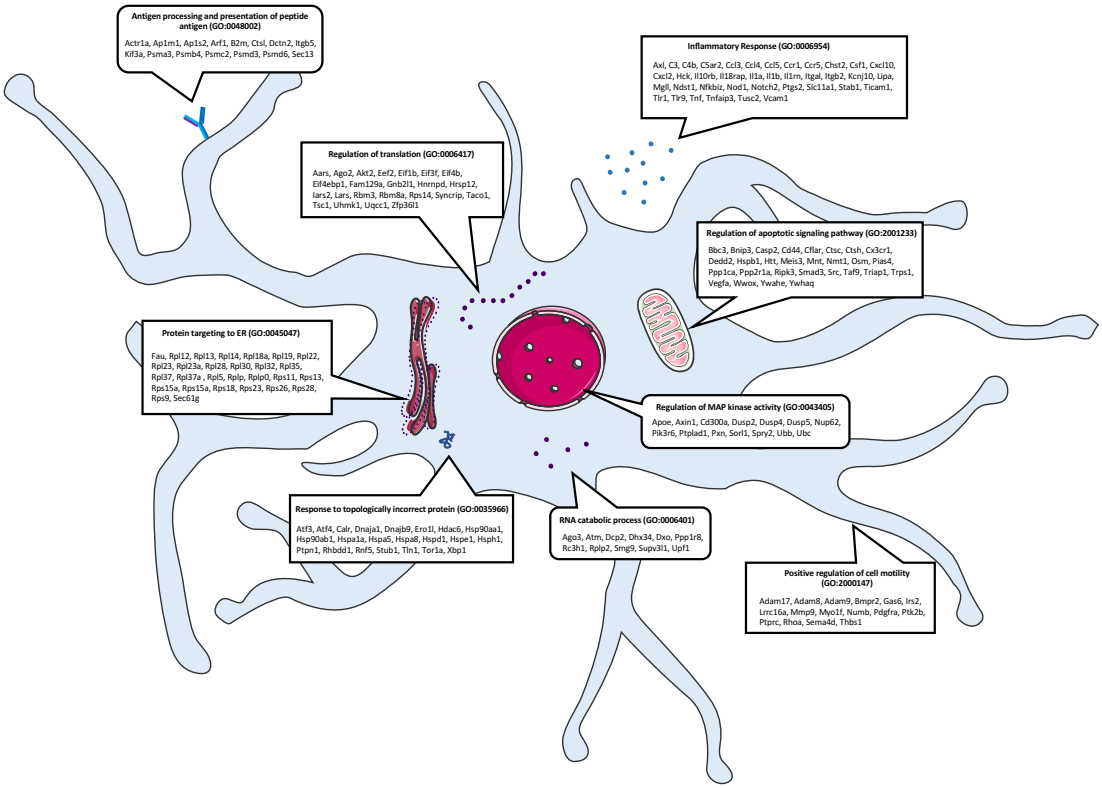


Figure 5.1: Altered biological processes in microglia 1 week after LPS

Biological processes that LPS differentially expressed genes were enriched for ($p < .05$), with some of the genes that are driving the ontologies (overlapping genes were removed, but several genes drove multiple ontologies).
Figure created using http://servier.com/Powerpoint-image-bank

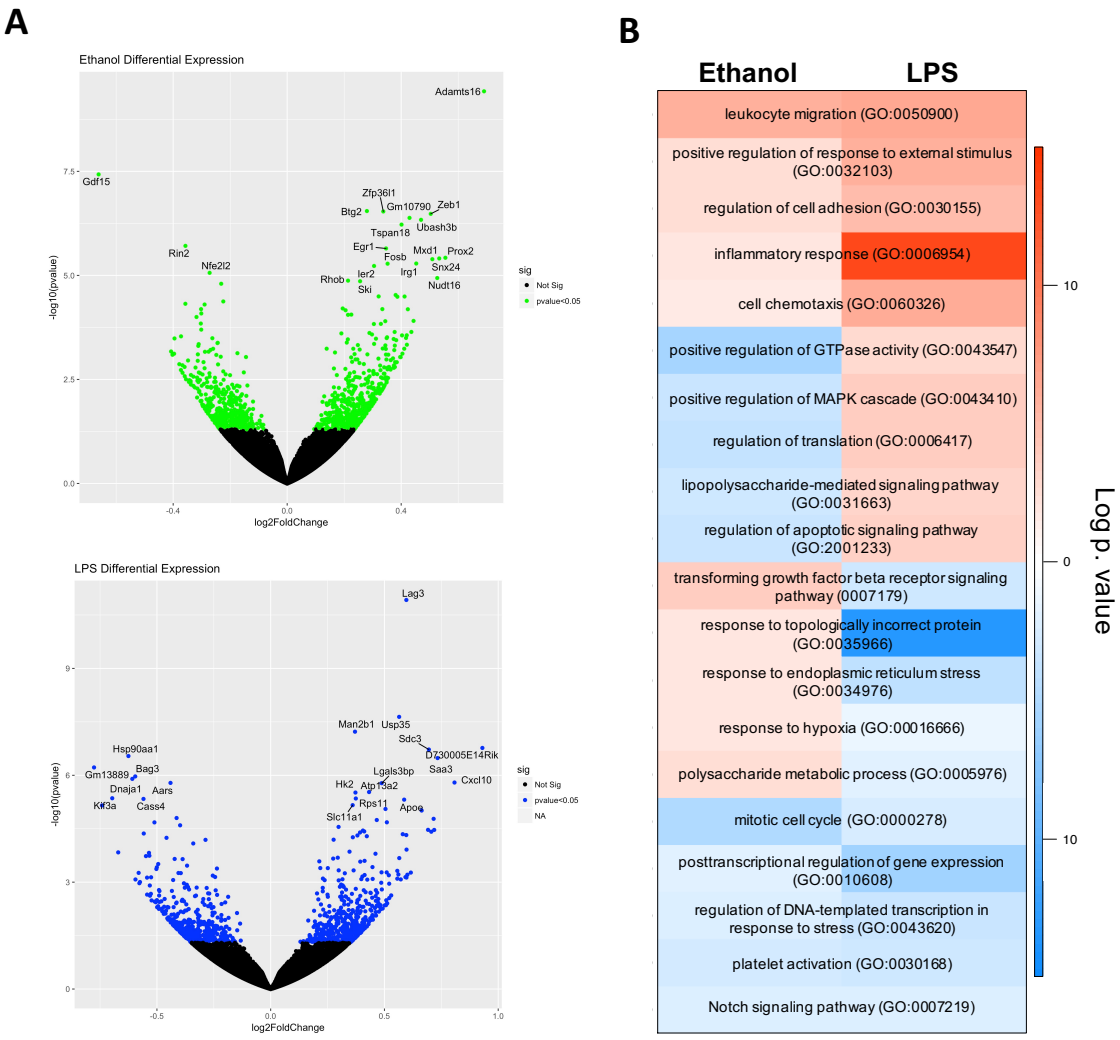


Figure 5.2: Microglial differential expression after LPS and ethanol treatments

Comparison of differential expression in microglia following LPS and Ethanol treatments. **A.** Volcano plots showing differential expression by $\log_2$ fold change vs. $\log_{10}$ p value. Colored dots have a p value < .05 and the top 20 differentially expressed genes (by p value) are labeled. **B.** Enriched Biological Processes based on significantly upregulated genes (red) or downregulated genes (blue) within each treatment. Color intensity is based on $\log_{10}$ p value (darker colors correspond to lower p values), while red or blue indicates whether the genes were up or down regulated. All ontologies met a p value of < .05.

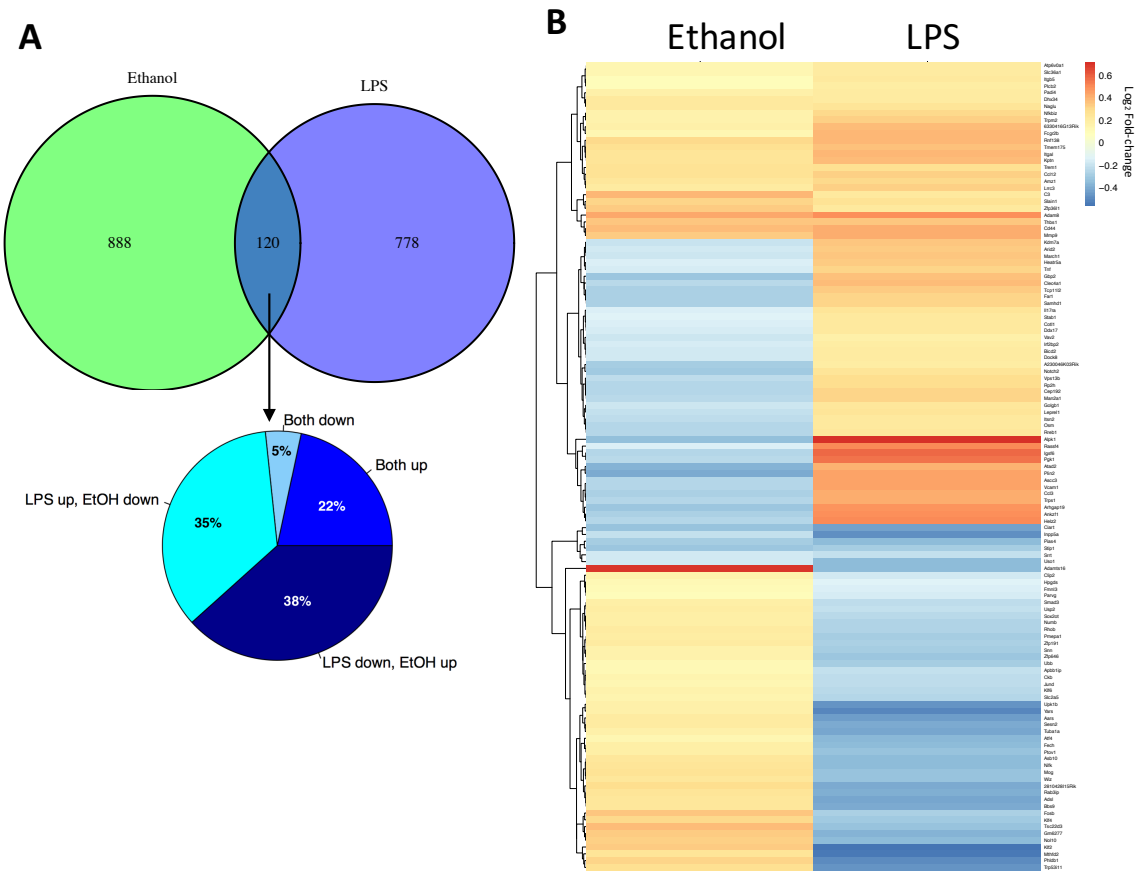


Figure 5.3: Microglial gene expression overlap between LPS and ethanol

Comparison of the differentially expressed genes that overlap between Ethanol and LPS. **A.** Ethanol resulted in 1008 differentially expressed genes and LPS resulted in 898 differentially expressed genes. The overlap between the two groups is 120, which is greater than expected by chance (2.2e-16, Fisher's test). Analysis of the direction of fold change within this overlap shows that only 27% of the changes are occurring in the same direction. **B.** Heat map showing expression of the 120 overlapping genes. Color intensity indicates $\log_2$ fold change, with red being upregulated and blue being downregulated. Gene symbols are shown on the right.

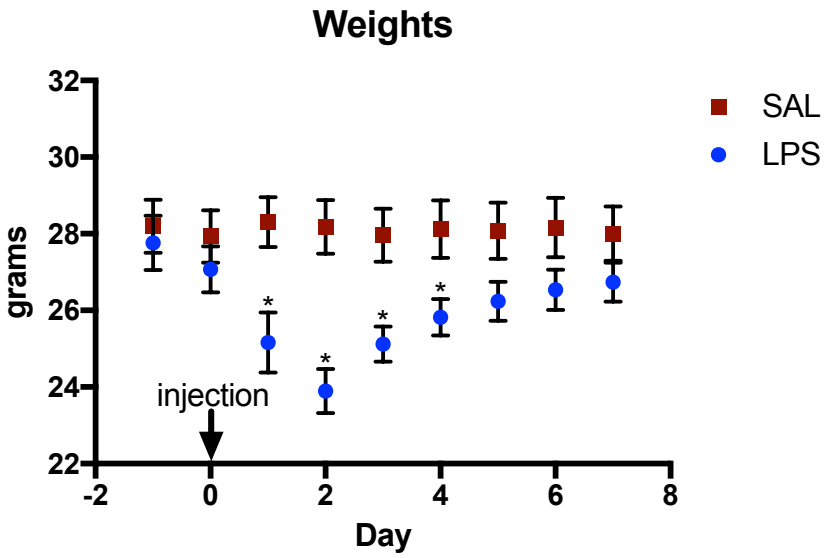


Figure 5.4: Verification of LPS effect

Data verifying the effect of LPS treatment. The mice were treated with LPS or saline and sacrificed 1 week later. Weights were measured 1 day before injection and each subsequent day until sacrifice. The LPS group showed significantly lower weight compared to the saline group on days 1-4 ($p < 0.05$, t-test). Weight increased after day 2 and was not significantly different from the saline group on day 7.

5.VIII. TABLES

Description	# of genes	GO Biological Process	p value	Reactome	p value
Microglia LPS-EtOH	**120**	positive regulation of response to wounding (GO:1903036)	1.35E-05	Integrin cell surface interactions_Homo sapiens_R-HSA-216083	5.13E-05
Same Direction	**32**	extracellular matrix organization (GO:0030198)	1.96E-05	Integrin cell surface interactions_Homo sapiens_R-HSA-216083	3.85E-06
up in both	26	extracellular matrix organization (GO:0030198)	5.45E-06	Extracellular matrix organization_Homo sapiens_R-HSA-1474244	1.38E-06
down in both	6	transcytosis (GO:0045056)	2.70E-03	Synthesis of IP2, IP, and Ins in the cytosol_Homo sapiens_R-HSA-1855183	3.30E-03
Opposite Directions	**88**	cellular response to cytokine stimulus (GO:0071345)	2.27E-04	Regulation of TNFR1 signaling_Homo sapiens_R-HSA-5357905	3.38E-04
LPS up, EtOH down	42	positive regulation of inflammatory response (GO:0050729)	3.45E-05	Cytokine Signaling in Immune system_Homo sapiens_R-HSA-1280215	1.76E-03
EtOH up, LPS down	46	regulation of transcription from RNA polymerase II promoter in response to stress (GO:0043618)	1.14E-04	TGF-beta receptor signaling activates SMADs_Homo sapiens_R-HSA-2173789	5.39E-05
Total Homogenate LPS-EtOH overlap *	**104**	synaptic transmission (GO:0007268)	8.85E-05	Neurotransmitter Receptor Binding And Downstream Transmission In The Postsynaptic Cell_Homo sapiens_R-HSA-112314	9.88E-05
Same Direction	**86**	histone H3 deacetylation (GO:0070932)	9.65E-05	Neurotransmitter Receptor Binding And Downstream Transmission In The Postsynaptic Cell_Homo sapiens_R-HSA-112314	3.40E-05
up in both	52	histone H3 deacetylation (GO:0070932)	2.13E-05	Cooperation of PDCL (PhLP1) and TRiC/CCT in G-protein beta folding_Homo sapiens_R-HSA-6814122	1.77E-04
down in both	34	blood vessel development (GO:0001568)	9.17E-03	Glutamate Binding, Activation of AMPA Receptors and Synaptic Plasticity_Homo sapiens_R-HSA-399721	1.27E-03
Opposite Directions	**18**	activation of signaling protein activity involved in unfolded protein response (GO:0006987)	2.46E-05	Unfolded Protein Response (UPR)_Homo sapiens_R-HSA-381119	5.98E-05
LPS up, EtOH down	10	activation of signaling protein activity involved in unfolded protein response (GO:0006987)	4.46E-04	Unfolded Protein Response (UPR)_Homo sapiens_R-HSA-381119	8.04E-04
EtOH up, LPS down	8	neurotransmitter-gated ion channel clustering (GO:0072578)	3.20E-03	Molybdenum cofactor biosynthesis_Homo sapiens_R-HSA-947581	2.40E-03

Table 5.1: Ontologies for ethanol-LPS gene overlap in microglia and total homogenate

GO Biological Process and Reactome ontologies for genes that overlap between ethanol and LPS microglia and genes that overlap between ethanol and LPS total homogenate. Ontologies are given for the entire set of overlapping genes, as well as those changed in the same and opposite directions (broken up by direction). *Total homogenate analysis is based on data from Osterndorff-Kahanek et al. 2013.

Chapter 6: Summary and Future Directions

6.I. Summary of Results

Despite their prevalence and impact, AUDs are largely undertreated, in part because few individuals seek treatment (only 20%) and in part because existing treatments aren't effective in preventing relapse (Grant et al. 2015). The emerging link between neuroimmune signaling and brain pathologies has revealed a role for immune signaling in alcohol-induced damage and dependence. Studies have shown that ethanol induces neuroimmune signaling, which further promotes consumption, suggesting that targeting the innate immune responses in the brain may have therapeutic potential. My work has provided important insight about which neuroimmune pathways are changing in response to chronic ethanol, and which glial cell types are contributing to these changes (**Fig. 6.1**).

Studies regarding the neuroimmune effects of alcohol have largely focused on the importance of TLR4, NF-κB activation, and cytokine production (G. Robinson et al. 2014). However, few studies have investigated the effects of ethanol on the two TLR4 signaling pathways, the MyD88-dependent pathway and the TRIF-dependent pathway. We hypothesized that because the MyD88-dependent pathway signals to NF-κB and is utilized by almost every TLR, components of this pathway would change with ethanol. However, in chapter 2, I showed that many of the gene expression changes in the PFC were occurring in the TRIF-dependent pathway, the pathway that increases expression of interferons and interferon-inducible genes. *Cxcl10*, an interferon inducible gene produced

in response to TRIF-pathway signaling, was also increased, suggesting that the pathway is not just increased, but also activated.

Going against the idea that TLR4 is central to the ethanol-immune response, I showed that *Tlr3,* an endosomal TLR which signals exclusively through the TRIF-dependent pathway, was increased more than *Tlr4*. In addition, I showed that ethanol increased expression of *Tlr7*, another endosomal TLR that can activate interferons, and its downstream transcription factor *Irf7*. Collectively, these data suggested that chronic ethanol alters TRIF-dependent signaling in the PFC. In addition, because we looked at 2 time-points and multiple brain regions (PFC, nucleus accumbens, amygdala), we identified brain-region- and time- dependent changes in TLR expression. These changes emphasized that most TLR changes occur in the PFC 24-hours after ethanol removal, but that some similar changes occur in the accumbens, while changes seem to be in the opposite direction in the amygdala. While previous data has shown an ethanol-induced TRIF-pathway response *in vitro*, this study was the first to provide evidence of the pathway changing *in vivo*. Additionally, this was the first study to show that rodent voluntary ethanol consumption, which has minimal effects on glial markers, can change TLR and cytokine expression. Furthermore, we showed that a TRIF-pathway inhibitor decreased ethanol consumption, suggesting a causal role of this signaling in the regulation of drinking behavior.

Because my work in chapter 2 suggested that TRIF-dependent signaling may mediate the ethanol-neuroimmune response, we were interested in determining which CNS cell types express these genes and proteins. In chapter 3, I used isolated microglia

and astrocytes to determine the cell-type enrichment of TLR pathway genes with and without immune activation. I discovered that most of the TLR pathway genes were enriched and changed in microglia, particularly cytokines. However, I did notice some differences in cell-type expression within the same pathway, suggesting that these genes may be involved in other pathways in non-microglial cells. In addition, I determined that *Tlr3* expression is most enriched in astrocytes, and that astrocyte expression increases following LPS. In combination with astrocyte enrichment of *Irf3*, these results suggest a possible role for TRIF-dependent signaling in astrocytes. Although I was unable to determine the protein cell-type localization due to technical problems, I did determine that many of TLR antibodies are non-specific and several of these antibodies show no protein expression in microglia. Furthermore, different antibodies to the same protein give different localization results. These data highlight the reasons for disagreement over protein localization in the neuroimmune field and provide rationale for using alternative methods to determine protein localization.

After determining that some, but not all, of the ethanol-induced gene expression changes from chapter 2 were likely coming from microglia, I sought to further elucidate the microglial response to ethanol. Because ethanol-induced gene expression changes *in-vivo* are measured using a mix of cell types, it was unclear how microglia changed following chronic ethanol consumption. In chapter 4, I used isolated PFC microglia and revealed that most ethanol-induced microglial changes are not detected in a typical gene expression preparation (e.g. total homogenate). Furthermore, I found that ethanol increased expression of a network of genes related to endosomal TLR signaling and

TGF-β signaling. These results not only validate the results from chapter 2, but also suggest an important role for the cytokine TGF-β in the ethanol neuroimmune response. TGF-β has recently emerged as an important mediator of microglial function (Butovsky et al. 2013), and has been implicated in peripheral immune responses to ethanol (Dooley & Dijke 2012; Kim et al. 2009), but has not been studied regarding the ethanol-neuroimmune response. In addition, the lack of cytokine gene expression changes, suggested that the microglial response to ethanol differs from the typical pro-inflammatory response to immune activators like LPS. To further evaluate these differences, I profiled the microglial response to LPS in chapter 5.

Because our results in chapter 3 suggested that the microglial response to LPS early on includes increased expression of pro-inflammatory cytokines that were unchanged in microglia by ethanol, we decided to evaluate the microglial response 1 week after LPS exposure. Previous work from the lab revealed that PFC gene expression changes 1 week after LPS were similar to changes induced by chronic ethanol (Osterndorff-Kahanek et al. 2013). However, my work in chapter 5 revealed that the effects of LPS and ethanol on microglia are very different. These results suggest that some of the overlapping effects are due to other cell types, not microglia.

Collectively, my dissertation reveals novel ideas about how ethanol is altering neuroimmune signaling and the microglial response. Specifically, I discovered that endosomal TLRs and interferon producing pathways respond to chronic ethanol and mediate consumption. At a time where manipulations of TLR4 have failed to change

consumption (Harris et al. 2017), this finding presents a new pathway to explore. In addition, I found that microglial specific changes in response to chronic ethanol involve altered expression of the TGF-β signaling pathway. Given the importance of TGF-β in microglial function and cross-talk with other CNS cell types (Koeglsperger, Li, Brenneis, Saulnier, Mayo, Carrier, Selkoe & Weiner 2013a; Lodge & Sriram 1996; C. P. Chen et al. 2006), these data present a rationale to further study the role of this pathway in the ethanol neuroimmune response. Finally, I showed that the microglial response to ethanol is different from the LPS response *ex vivo*, highlighting the unique immunomodulatory function of alcohol in the CNS. These results provide new evidence about how alcohol changes neuroimmune signaling but also leave us with many unanswered questions. Therefore, several studies that will expand on my work are already ongoing. Specifically, future experiments will be determine the role of endosomal TLR signaling in regulating alcohol consumption as well as revealing the brain-region specific and cell-type specific contributions. Other studies should elucidate the role of microglial function in the ethanol-induced immune response. Lastly, future work will uncover the role of TGF-β signaling in the microglial response to ethanol, and use drugs targeting this pathway to modulate ethanol consumption.

6.II. Future Directions

6.II.a. The role of TLR3/TRIF signaling in ethanol consumption

My work in chapter 2 revealed increased expression of endosomal TLRs, TRIF-dependent signaling, and interferon inducible genes following ethanol consumption. While inhibition of the TRIF-dependent pathway decreased consumption, these results do not explain how these pathways are changing or how they regulate ethanol consumption.

6.II.a.i. How alcohol alters TLR3/TRIF/Interferon signaling

Studies regarding the neuroimmune effects of alcohol suggest that alcohol induces innate immune signaling both through peripheral and direct CNS effects. To better understand and target specific immune changes, it is necessary to understand the mechanism by which they occur. One way to determine if the observed CNS changes are a result of peripheral immune signaling is to inject cytokines that are induced peripherally in response to ethanol, such as TNF-α and IL-1β. If peripheral cytokines can induce the same changes that occur with alcohol consumption, the effect is likely indirect and should be targeted in the periphery. However, studies also suggest that ethanol directly induces neuroimmune signaling in the brain, via the production of endogenous ligands and the clustering of TLRs into lipid rafts (Fernandez-Lizarbe et al. 2008; Coleman et al. 2017; Whitman et al. 2013).

Interestingly, Consuelo Guerri's group has shown that adding ethanol to microglial cultures leads to activation of the TRIF-dependent pathway (Fernandez-

Lizarbe et al. 2009). Another study using astrocyte cultures overexpressing TLR4, revealed that ethanol induces TLR recruitment to lipid rafts and internalization to endosomes, and that TRIF-dependent signaling is dependent on clathrin-mediated endocytosis (Pascual-Lucas et al. 2014). Both studies suggest that ethanol can directly induce TLR4-TRIF dependent signaling in microglia and astrocytes, however, they did not measure changes in TLR3. Furthermore, these studies conclude that the microglial induction of TRIF-dependent signaling is entirely TLR4 dependent, but did not look at the effects of TLR4 knockout on TRIF-signaling in astrocytes. Therefore, TLR3 induced TRIF signaling could be occurring in astrocytes in response to ethanol. Future studies using primary cultures could better elucidate the direct effects of ethanol on TLR4-TRIF signaling and TLR3-TRIF signaling in astrocytes and microglia. Because of the overlap between the two pathways, knockout of TLR3 or TLR4 could be used to determine which pathway is responsible.

Additional work has suggested that ethanol causes the release of endogenous TLR ligands, including HMGB1 and miRNAs (Coleman et al. 2017; Whitman et al. 2013; Crews et al. 2013). qPCR analysis could be used to determine whether our voluntary ethanol paradigm produced changes in expression of these molecules or other endogenous TLR ligands. If these molecules are increased in response to voluntary ethanol, glial cultures or slice cultures could be used to further investigate whether these molecules activate TRIF-dependent signaling in the brain. Additionally, direct infusion of these ligands into the brain could determine whether they alone can induce the changes

seen in response to chronic ethanol. Conversely, antagonists or siRNAs could be used to see if preventing activity of these ligands prevents gene expression changes.

6.II.a.ii. How TRIF-dependent signaling regulates consumption

My results from chapter 2 suggest that TRIF-dependent signaling increases in response to chronic ethanol, and that changes in TRIF-signaling regulate drinking behavior. However, due to the myriad of changes we observed, it remains unclear which TLRs, brain regions, and cell types are mediating this response.

To determine which TLR is responsible for the changes, knockout animals can be used, and in part this has already been done. Yuri Blednov has showed that TLR4 knockout mice do not decrease consumption (Harris et al. 2017), while MyD88 knockout mice increase consumption (Blednov et al. 2017), and CD14 (Blednov et al. 2017) and TLR3 knockout mice decrease consumption (unpublished data). These results suggest that TLR3 signaling increases consumption, while MyD88 signaling decreases consumption. However, these studies have not measured brain gene expression changes following consumption to see how they are changed, therefore it is not clear whether these knockouts are modulating drinking by changing TRIF-dependent gene expression. Furthermore, although TLR3 primarily signals through the TRIF-dependent pathway, it can also activate NF-κB through TRAF6 (Kawai & Akira 2010). Therefore, to determine whether TRIF-dependent signaling specifically is responsible, knockout mice for components of the TRIF-dependent pathway need to be studied.

The Blednov/Messing group plans to obtain and test mice that are null mutants for components of the TRIF pathway and the interferon response (TRIF, IKKε, and IFNAR1) as part of the INIA consortium grant (2U01AA013520-16). Additionally, it would be interesting to investigate whether knockout of *Lgmn* impacts drinking. In chapter 4, *Lgmn* expression was changed in microglia in response to ethanol and was correlated with ethanol consumption. *Lgmn* encodes the protein endopeptidase, which is involved in the cleavage of endosomal TLRs, and could therefore regulate activation of multiple TLRs (Sepulveda et al. 2009). Furthermore, cathepsins are also involved in the cleavage of endosomal TLRs, and mutant mice null for cathepsin f and s decrease ethanol consumption (Blednov, Ponomarev, et al. 2011). Therefore, cleavage of TLRs might represent an important point of regulation. Lgmn/AEP inhibitors also exist and could be tested in ethanol consumption paradigms.

In addition to measuring alcohol consumption, gene expression analysis could be performed on mutants after ethanol consumption and compared to wild type littermates to see if the gene knockout impacts ethanol-induced changes. Furthermore, functional consequences of neuroimmune signaling could be studied (e.g. neuronal death, decreased neurogenesis, hyperexcitability, changes in neurotransmitter and neuropeptide levels). Behavioral changes related to cortical function (anxiety, impulsivity, learning and memory) could also be evaluated in mutant mice in response to ethanol and compared to wildtype littermates.

Although global knockout mice have been effective in determining genes that influence consumption, they provide little insight into the mechanism by which they alter

drinking behavior. Because of the widespread effects of alcohol on immune-signaling, global mutants make it difficult to determine whether the behavioral response is due to the periphery or the CNS. These distinctions become important not only for understanding the changes, but also for developing treatments that need to cross the blood-brain barrier. In addition, gene knockouts during development can result in compensatory signaling of other pathways, possibly distorting results. There is also evidence of global knockouts not changing drinking, but brain-region specific knockdown producing effects (Harris et al. 2017; Juan Liu et al. 2011). Therefore, CNS specific manipulations would be useful to understand whether the PFC is responsible for changes in behavior. One way to go about doing this is using viral vectors to deliver shRNAs or cre-recombinase into the brain region of interest (Truitt et al. 2016). This method additionally has the advantage and drawback of cell-type specificity. Viral vectors targeting a specific cell type can be helpful when trying to determine the cell-type specific contributions. However, this can also present a challenge because microglia are notoriously difficult to target with viral vectors.

In addition to using viral vectors, conditional knockout mice could be used to investigate cell-type specific effects. For example, recent studies profiling brain cell types have identified microglial and astrocyte specific genes. Mouse lines could be developed with cre under control of these cell type-specific promoters and could be crossed with floxed mice to create cell-type specific mutants. These experiments would further our understanding of how different brain cell-types contribute to the immune ethanol response *in vivo*.

If reduction of TLR3/TRIF signaling should reduce ethanol consumption, then activation should increase consumption. Consistent with this idea, recent work in the lab has revealed that injection of the TLR3 ligand Poly I:C alters brain gene expression and increases ethanol consumption. Additionally, work profiling the response in TLR3 and MyD88 knockout aims to determine the mechanism of change. Although TLR3 and TRIF-signaling are the primary target, results in chapters 2 and 4 suggest a potential role for other endosomal TLRs. In keeping with this, the effect of a TLR7/8 agonist is also currently being investigated.

6.II.b. Glia and ethanol consumption

6.II.b.i. Localization of TLR proteins

One of the goals of chapter 3 was to determine the CNS cell-type localization of TLR pathway genes and proteins. However, due to non-specific antibodies, I was unable to make any conclusions. I hope that someone considers my findings about antibodies and uses a different approach to determine cell-type localization in the CNS. As I mentioned in chapter 3, a proteomic approach using isolated cells may be better suited to answer this question. Additionally, development of transgenic mice with a GFP tagged gene would be useful for this purpose. Although I suspect that protein data collected using non-antibody techniques would mirror my qPCR results, it is important that this be determined conclusively.

6.II.b.ii. Effects of microglial depletion on microglial immune response

Recent studies have developed the ability to deplete microglia from the adult mouse brain (Asai et al. 2015). This technique has been used to reveal the role of microglia in CNS pathology and could be useful in understanding how microglia influence the ethanol response. Currently, the lab is planning to use microglial depletion (Asai et al. 2015) to see how it changes ethanol consumption. In addition, it would be insightful to evaluate gene expression changes following ethanol exposure in the microglial-depleted brain. If microglia are responsible for the neuroimmune changes we are observing, we would expect those changes to go away. In contrast, if other cells are contributing to the neuroimmune response, changes would be detectable in the microglia-depleted brain.

Furthermore, if microglial depletion does change ethanol consumption, it would be important to determine the effects of microglial depletion on ethanol changes in brain activity (e.g. neurotransmitter activity, synaptic plasticity, neurogenesis, neuronal damage) and ethanol induced changes in cortical function.

6.II.b.iii. Effects of alcohol on microglial immune response and function

Although my gene expression data in chapter 4 provided some insight into the ethanol-induced microglial changes, any functional consequences are speculation. Recent studies suggest that certain disease states, such as Alzheimer's, reflect a change in the microglial response to stimuli (Bernhardi et al. 2015). Furthermore, studies using isolated immune cells from alcoholics have found changes in immune cell response to TLR

ligands (Leclercq, De Saeger, et al. 2014) (Szabo & Saha 2015). Therefore, I think it would be informative to culture microglia from mice that had undergone ethanol consumption. Then, the cells can be exposed to stimuli *in vitro* and any functional changes can be determined. Many processes could be evaluated, including cytokine and ROS production, phagocytosis, and migration and invasion. In addition, other cell types (neurons, astrocytes, oligodendrocytes, etc.) could be co-cultured or cultured in conditioned microglial media, to determine the effect of microglial changes on other CNS cells. These experiments would help to elucidate how microglial function changes in response to chronic ethanol consumption, and how microglia could be mediating some of the detrimental effects of alcohol consumption.

6.II.b.iv. Microglial changes and CNS cross-talk

The results I presented in chapter 4 highlight the microglia specific changes that occur in response to ethanol *in vivo*. However, one of the reasons for evaluating these changes *in vivo* is that the neuroimmune response in the brain is the product of cross-talk between microglia and other cell-types. Thus, it would be useful to isolate multiple cell types from the same ethanol-exposed mouse and profile the gene expression changes. Currently, the lab has collected both microglial and astrocyte gene expression data from ethanol-exposed mice. Analysis of these data should provide interesting insight about how the glial responses differ and potentially highlight some cross-talk signals that are perturbed.

6.II.c TGF-β signaling and the neuroimmune response to ethanol

My results in chapter 4 suggest that microglial TGF-β signaling is perturbed in response to chronic ethanol. Given the role of TGF-β in microglial function, alcoholic liver diseases, and CNS cross-talk, I believe this pathway warrants further investigation.

6.II.c.i. Further evaluate changes in TGF-β pathway expression

Although my microglial data suggest that TGF-β signaling is perturbed in microglia in response to ethanol, the next logical step would be to investigate this further. There are multiple TGF-β ligands and receptors and they vary in their cell type expression. qPCR analysis could be used to determine if changes in the TGF-β pathway are detected in the total homogenate from the PFC and other brain regions. In addition, immunohistochemistry could be used to look at protein level changes within the CNS cell types. Rodent models are a good resource for evaluating ethanol-induced changes, particularly using isolated cell preparations that require live cells. However, it is important to know that the changes observed in rodent models are also observed in humans. Therefore, it would be worthwhile to also look at human-postmortem tissue to see if there are any changes in TGF-β.

6.II.c.ii. LINCS analysis and drugs that target the microglial response

The Library of Network Based Cellular Signatures (LINCS) program uses genomic signatures to identify drugs and compounds that could normalize the gene

expression changes seen with a particular disease or disorder (Hurle et al. 2013; Lamb et al. 2006). The LINCS project has tested approximately 20,000 drugs and compounds on several different cell lines and has collected gene expression data. In order to make this process high throughput, LINCS only profiles gene expression for 978 landkmark genes which they call they call the landmark 1000, but use this gene expression information to infer expression of other genes.

With the help of Laura Ferguson, we input the ethanol microglial gene expression data to identify drugs that would either mimic or oppose the ethanol-induced genomic signature. We tried two different methods for inputting the microglial data, one using the top 100 differentially expressed genes in microglial module 3, and the second using the differentially expressed genes in microglial module 3 that were also landmark 1000 genes. Using the overlap between these two searches, we identified several drugs that should act as a strong negative or a strong positive. In theory, a strong negative drug would produce the opposite gene expression changes that what we observed and would restore homeostasis, while a strong positive drug would mimic our ethanol-induced gene expression changes, thus exacerbating the phenotype. The top 10 strong negative and strong positive results are shown in **Figure 6.2**.

Based on the microglial gene expression changes we observed in response to chronic ethanol, we identified several drugs that would theoretically normalize gene expression. These drugs included a TGF-β/p38 MAPK inhibitor (LY-364947), a JNK inhibitor (CG-930), and FGFR and VEGFR inhibitor (brivanib), and a MEK/MAPK inhibitor (PD-0325901), all of which are currently involved in clinical trials. Future

studies could use these drugs in rodent consumption models and determine whether the drug normalizes gene expression changes and decreases ethanol consumption.

6.II.c.iii. TGF-β mutant mice

Recently, Koeglsperger et al. developed a mutant mouse line lacking TGF-β1 specifically in the CNS. They found that this mouse displayed hippocampal neuronal loss, decreased astrocyte glutamate transporter expression and uptake, and GluN2B-dependent aberrant synaptic plasticity (Koeglsperger, Li, Brenneis, Saulnier, Mayo, Carrier, Selkoe & Weiner 2013b). Given the role of glutamate signaling in neuroimmune-induced damage and synaptic activity, it would be interesting to further study this mouse. Future studies could look more closely at the PFC in these mice to see if there are similar dysfunctions. In addition, using these mice for ethanol consumption experiments could elucidate the role of TGF-β signaling in regulating drinking behavior. If CNS-TGF-β1 mutant mice show changes in ethanol consumption, the effects of ethanol on pro-inflammatory environment, brain activity, and cortical function could also be evaluated.

6.II.d. Effects of Neuroimmune signaling on Neuotransmission

Within our lab, the primary focus is on identifying gene expression changes and then using these changes to identify drug targets for regulating ethanol consumption. Thus, many of the future studies I have proposed mostly involve determining whether genetic or pharmacological manipulation change ethanol consumption (**Fig. 6.3**). However, it is also

important to determine how altering neuroimmune signaling changes ethanol consumption. As I described in Chapter I.V.b., there are several proposed mechanisms by which altered neuroimmune signaling changes neuronal activity and therefore changes behavior. Although these changes are not necessarily a focus of our lab, it is important for future studies to elucidate these mechanisms. Therefore, any genetic or pharmacological manipulations that are found to alter drinking behavior, could be further studied to determine how neuronal activity is impacted. I have pointed out some of these potential studies in **Figure 6.3**.

6.II.e. Treatment in humans

Although cell culture and rodent models are important for discovering and understanding the role of neuroimmune signaling in ethanol consumption, the end goal is developing treatments for humans. Thus, the long-term future goal with regards to this project, is understanding the neuroimmune response well enough that we can identify a therapeutic treatment for use in humans.

6.III. FIGURES

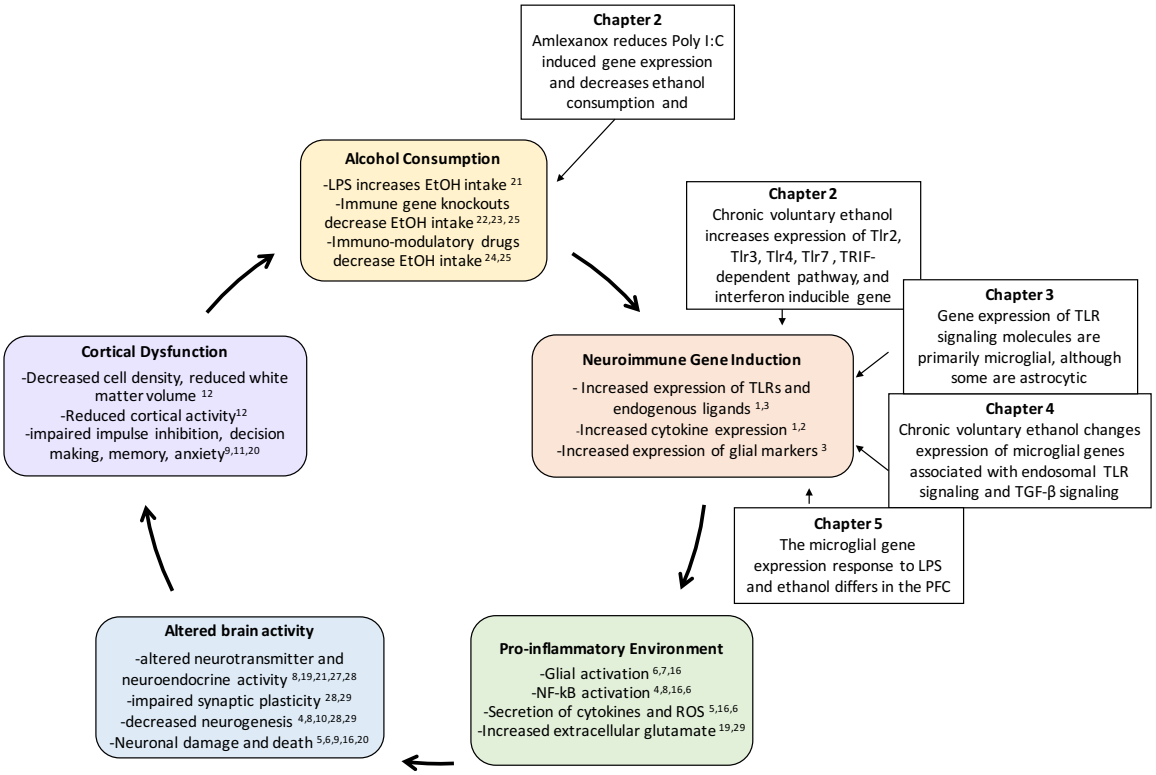
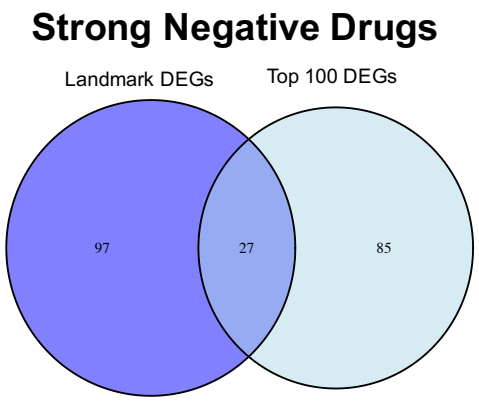
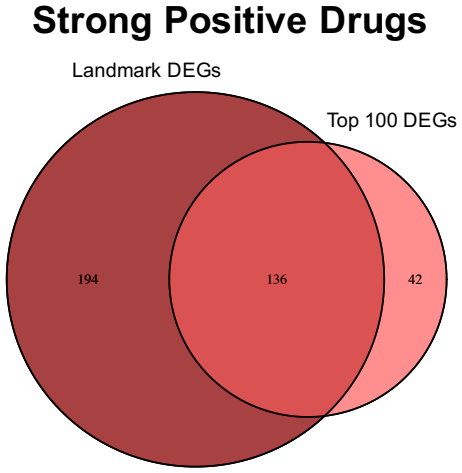


Figure 6.1: Summary of results within hypothesized cycle

The hypothesized cycle from chapter 1 of how alcohol changes neuroimmune signaling and thus regulates consumption behavior. Results from each chapter are shown with arrows indicating which part of the cycle these findings expand on. Most of the findings in this dissertation reveal how alcohol changes neuroimmune gene expression. Future studies should address how these gene expression changes influence the pro-inflammatory environment, brain activity, cortical function, and alcohol consumption.



Drug Name	Description	Top 100 Score	Landmark Score
LY-364947	TGF beta receptor inhibitor, p38 MAPK inhibitor	-99.26	-99.93
iloperidone	dopamine receptor antagonist, serotonin receptor antagonist	-99.72	-99.05
cobalt(II)-chloride	HSP agonist	-99.15	-97.22
VX-222	HCV inhibitor, RNA-directed RNA polymerase inhibitor	-98.2	-98.45
NECA	adenosine receptor agonist	-98.87	-97.29
CG-930	JNK inhibitor	-94.57	-99.4
demeclocycline	30S ribosomal subunit inhibitor	-95.1	-99.12
brivanib	alcohol dehydrogenase inhibitor, FGFR inhibitor, fibroblast growth factor receptor (FGFR) inhibitor, vascular endothelial growth factor receptor (VEGFR) inhibitor, VEGFR inhibitor	-99.12	-95.36
PD-0325901	MEK inhibitor, MAP kinase inhibitor, protein kinase inhibitor	-97.74	-97.56
hydroxycholesterol	LXR agonist, ABC transporter expression enhancer, alpha secretase activator, beta secretase inhibitor, glutamate receptor modulator	-98.54	-94.44



Drug Name	Description	Top 100 Score	Landmark Score
calyculin	protein phosphatase inhibitor	99.93	99.93
withaferin-a	acetylcholinesterase inhibitor, butyrylcholinesterase inhibitors, IKK inhibitor, NFkB pathway inhibitor, PKC inhibitor	99.44	99.47
KI-8751	PDGFR alpha and c-Kit inhibitor, vascular endothelial growth factor receptor 2 (VEGFR2) inhibitor, VEGFR inhibitor	98.51	99.61
QL-XII-47	BMX inhibitor, Bruton's tyrosine kinase (BTK) inhibitor, bruton's tyrosine kinase inhibitor, cytoplasmic tyrosine protein kinase BMX inhibitor	98.7	99.19
tacedinaline	HDAC inhibitor, cell cycle inhibitor	98.17	99.47
menadione	CDC inhibitor, mitochondrial DNA polymerase inhibitor, phosphatase inhibitor, pyruvate kinase isozyme inhibitor	98.56	99.15
HU-211	glutamate receptor antagonist, apoptosis stimulant, NFkB pathway inhibitor, reducing agent	98.17	99.15
homoharringtonine	apoptosis stimulant, protein synthesis inhibitor	98.52	98.94
calmidazolium	calcium channel blocker, calmodulin antagonist	99.26	98.66
alitretinoin	RXR agonist, ABC transporter expression enhancer, apoptosis stimulant, RAR agonist, retinoid receptor agonist	98.31	99.01

Figure 6.2: LINCS Analysis

Differentially expressed genes (DEGs) within MGM3 were used to query the LINCS-L1000 database, which contains the transcriptional responses of a diverse set of human cell lines in culture to thousands of chemical compounds. We constructed 2 different inputs – One comprised of the top 100 differentially expressed genes (based on fold change), and the other comprised of only the differentially expressed landmark genes. Landmark genes are those that are directly measured to construct the LINCS-L1000 database. We submitted these inputs using the clue.io website. A connectivity score is computed that assesses the similarity of the input and drug transcriptional signatures. A negative score indicates the drug has an opposing effect on gene expression, while a positive score indicated the drug has a similar effect on gene expression. The top 10 positive and negative scoring compounds are shown in the tables.

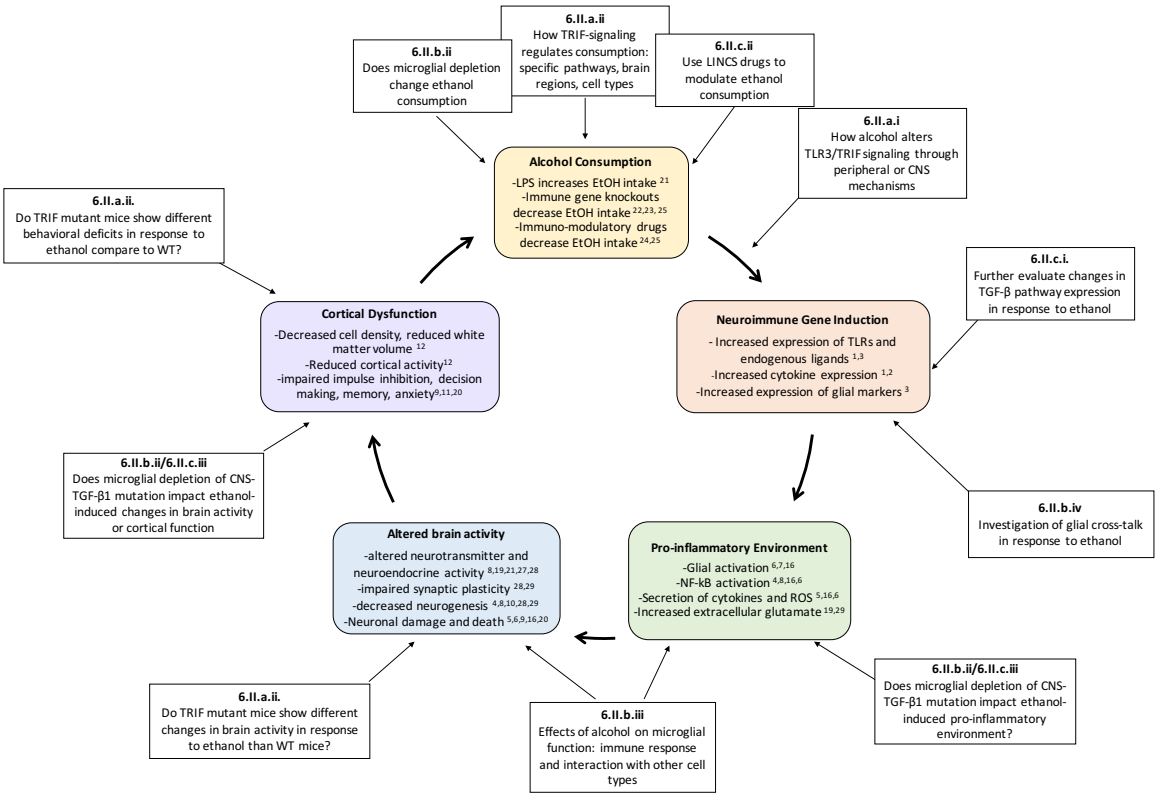


Figure 6.3: Summary of future directions within hypothesized cycle

The hypothesized cycle from chapter 1 of how alcohol changes neuroimmune signaling and thus regulates consumption behavior. Future directions are shown with arrows indicating which part of the cycle these findings expand on. Future studies aim to not only determine whether manipulation of gene expression changes alters consumption, but also how. To elucidate how, studies can investigate ethanol-induced changes in gene expression, pro-inflammatory environment, brain activity, and cortical dysfunction in mutant mice that show altered ethanol consumption.

Abbreviations/Glossary

Amlexanox: Inhibitor of IKKε and TBK1. Approved in the US as a topical paste used for the treatment of aphthous ulcer (canker sores).

AMY: *Amygdala*. Brain region that is part of limbic system and is involved in decision making and emotional reactions. Further divided into the central nucleus of the amygdala and the basolateral amygdala. The amygdala has reciprocal connections with the prefrontal cortex.

AP-1: *Activator protein 1*. Transcription factor that is a heterodimer of proteins Fos and Jun proteins. Activated in response to TLR signaling through the MAPK pathway. Leads to the transcription of pro-inflammatory cytokines as well as genes regulating differentiation, cell-cycle, and apoptosis.

AUD: *Alcohol use disorder*. DSM-5 term for alcohol dependence or abuse. Individuals meeting 2 out of 11 criteria during a 12-month period receive a diagnosis of AUD. AUD severity is based on the number of criteria met.

CCL2: *C-C motif chemokine ligand 2; also known as monocyte chemotactic and activating factor (MCP-1)*. Pro-inflammatory chemokine that is transcribed in response to activation of NF-κB. Considered a marker of M1 microglia. Signals through the receptor CCR2.

CCL5: *C-C motif chemokine ligand 5; also known as RANTES*. Chemokine that is transcribed in response to activation of the TRIF-dependent pathway and the IRF3 transcription factor.

CD11b: *Cluster of differentiation molecule 11b; Also known as integrin alpha M (ITGAM)*. Cd11b is expressed on immune cells and mediates adhesion, migration, and chemotaxis. Within the CNS, it is used as a microglial marker.

CD14: *Cluster of differentiation 14*. Pattern recognition receptor that acts as a co-receptor with TLR4 and MD-2 to detect LPS. There is also evidence that it acts as a co-receptor for TLR2, TLR3, TLR7, and TLR9.

COX-2: *Cyclooxygenase 2; also known as prostaglandin-endoperoxide synthase 2 (PTGS2)*. Enzyme that is involved in the conversion of arachidonic acid to prostaglandin. Released in response to TLR signaling and NF-κB activation.

CREB: *Cyclic AMP (cAMP) response element-binding protein*. Transcription factor that binds to cAMP response elements. CREB increases expression of genes like BDNF that promote neuronal survival.

CXCL10: *C-X-C motif chemokine 10, also known as Interferon gamma-induced protein 10 (IP-10)*. Chemokine secreted in response to interferon gamma, and in response to TRIF-pathway and IRF3 activation. Binds to chemokine receptor CXCR3.

CXCL12: *C-X-C motif chemokine 12; also known as stromal cell-derived factor 1 (SDF1)*. Chemokine that plays a role in neurogenesis and GABA release.

DAMP: *Damage-associated molecular pattern; also known as danger-associated molecular pattern*. Host molecules that are released

during cell damage or death and can be recognized by pattern recognition receptors and initiate an inflammatory response. Examples of DAMPs include HMGB1, heat shock proteins, extracellular matrix proteins, and S100 proteins. In addition, DNA and RNA outside of the cell can act as a DAMP.

ECM: *Extracellular matrix*. Extracellular molecules secreted by cells that provide structural support for the surrounding cells. The ECM is involved in cell adhesion, synapse formation, regulation of diffusion, and maintenance of blood brain barrier. ECM components are altered in response to neuroimmune signaling and can propagate the immune response by activating microglia.

EOD-2BC: *Every other day 2 bottle choice paradigm*. Rodent voluntary ethanol consumption paradigm that provides 24-hour access to both water and an ethanol solution every other day. Only water is provided on the off days. Every other day exposure leads to escalation in ethanol consumption over time.

GABA: *Gamma-aminobutyric acid*. GABA is the primary inhibitory neurotransmitter. Acute ethanol increases GABA activity, but long-term ethanol exposure leads to decreased GABA activity. GABA expression and activity can be regulated by cytokines.

HMGB1: *High mobility group box 1*. Chromatin protein that is secreted by immune cells. Can act as a ligand for TLR2, TLR3, TLR4 and RAGE.

HPA: *Hypothalamic-pituitary-adrenal axis*. Neuroendocrine system that involves interactions between three endocrine glands, the

hypothalamus, the pituitary gland, and adrenal glands. The HPA axis controls response to stress and immune signaling.

IBA1: *Ionized calcium binding adapter molecule 1; also known as Allograft inflammatory factor 1 (AIF1)*. Iba1 is specifically expressed in microglia within the brain and is upregulated with microglial activation.

IFNβ: *Interferon beta*. Type I interferon that binds to IFN-α/β receptor (IFNAR). Activated in response to TRIF pathway/IRF3 activation. Has antiviral activity.

IFNγ: *Interferon gamma*. Cytokine that is a member of the type II interferon family. IFN-γ causes macrophage/microglial activation into an M1 state and induces Class II MHC molecules.

IKKα: *Inhibitor of nuclear factor kappa-B (IκB) kinase subunit alpha*. Part of the IKK enzyme complex (IKKα, IKKβ, IKKγ) that is activated in response to the MyD88-dependent pathway. The IKK complex phosphorylates IκB (inhibitor of NF-κB) leading to NF-κB activation. It is also involved in the MyD88-dependent interferon response.

IKKβ: *Inhibitor of nuclear factor kappa-B (IκB) kinase subunit beta*. Part of the IKK enzyme complex (IKKα, IKKβ, IKKγ) that is activated in response to the MyD88-dependent pathway. The IKK complex phosphorylates IκB (inhibitor of NF-κB) leading to NF-κB activation.

IKKε: *Inhibitor of nuclear factor kappa-B (IκB) kinase subunit epsilon; also known as IKKI*. Interacts with TBK1 to phosphorylate IRF3.

IL-1β: *Interleukin 1 beta*. Pro-inflammatory cytokine that is transcribed in response to NF-κB activation. Exists as a precursor that is cleaved to form mature and active form. Binds to the IL1 receptor.

IL-4: *Interleukin 4*. Anti-inflammatory cytokine that induces macrophages and microglia into an M2 state.

IL-6: *Interleukin 6*. Pro-inflammatory cytokine that is transcribed in response to NF-κB activation.

IL-10: *Interleukin 10*. Anti-inflammatory cytokine that can block NF-κB activation. IL-10 expression can induce microglia and macrophages to a M0 state and serves as a marker of M0/M2 activation states.

IL-13: *Interleukin 13*. Anti-inflammatory cytokine that induces a M2 state.

iNOS: *Inducible nitric oxide synthases*. Enzyme involved in the production of free radical nitric oxide.

IRAK1: *Interleukin-1 receptor-associated kinase 1*. Enzyme involved in the MyD88-dependent pathway. Activated in response to phosphorylation by IRAK4. Interacts with TRAF6 resulting in activation of NF-κB.

IRAK4: *Interleukin-1 receptor-associated kinase 4*. Recruited in the MyD88-dependent pathway and phosphorylates IRAK1 leading to downstream activation of NF-κB.

IRF3: *Interferon regulatory factor 3*. Transcription factor activated by the TRIF-dependent pathway. Activation results in transcription of type I interferons and interferon inducible genes.

IRF7: *Interferon regulatory factor 7*. Transcription factor activated by the MyD88-dependent pathway in response to endosomal TLR

signaling. Transcribes type I interferons, particularly interferon alpha.

Lgmn: *Gene encoding legumain, also known as asparaginyl endopeptidase (AEP)*. Lysosomal protease that cleaves asparaginyl bonds. It is responsible for the cleavage of TLR9, leading to its activation in dendritic cells. It also appears to be involved in cleavage of TLR3 and TLR7. It may also be involved in the processing of peptides for MHC class II antigen presentation.

LPS: *Lipopolysaccharide; also known as lipoglycan or endotoxin*. Large molecules composed of lipid and polyscaccharides that are the major component of the outer membrane of gram-negative bacteria. LPS acts as a ligand for TLR4.

LTD/LTP: *Long-term depression/potentiation*. Activity-dependent reduction or increase in synaptic strength. Together LTD and LTP impact synaptic plasticity and therefore learning and memory.

M0: *Macrophage/microglial acquired de-activation state; also known as homeostatic state, M2c*. Macrophage and microglial state thought to represent *in vivo* homeostatic state or acquired deactivation of inflammatory response. Induced in response to TGF-β, IL-10, or apoptosis. Still not clearly defined, but markers are thought to be similar to M2 markers**.**

M1: *Macrophage/microglia classical activation state*. The classical macrophage or microglial activation state, induced by IFN-γ or LPS. Characterized by secretion of pro-inflammatory cytokines and reactive oxygen species and antigen presentation.

M2: *Macrophage/microglial alternative activation state; also known as M2a*. Macrophage/Microglial alternative activation state induced by IL-4 and IL-13. Characterized by secretion of anti-inflammatory cytokines and growth factors. Involved in the repair process after a pro-inflammatory response, including extracellular matrix repair and phagocytosis of debris.

MAL: *MyD88-adapter-like protein; also known as Toll-Interleukin 1 Receptor (TIR) domain-containing adapter protein*. Adapter protein required in addition to MyD88 for TLR2 and TLR4 signaling.

MAPK: *Mitogen-activated protein kinase*. Family of kinases involved in response to pro-inflammatory cytokines. MAPK signaling is activated in response to the MyD88-dependent pathway, TGF-β signaling, and TNF-α signaling. MAPK signaling leads to activation of the AP-1 transcription factors. Activation of MAPK can impact many cellular processes like apoptosis, cell cycle, inflammation, and neurotransmitter regulation.

MCP-1: *See CCL2*.

MHCI: *Major histocompatibility complex class I*. Class of molecules found on cell surface of all nucleated cells. MHC Class I molecules present non-self peptides from within the cell to T-cells. Activated microglia can display MHC class I proteins.

MHCII: *Major histocompatibility complex class II*. Class of molecules found on antigen-presenting cells. Antigen presentation involves phagocytosis and digestion of extracellular proteins which results in peptide fragments attached to the MHC II molecules. During

homeostasis, microglia do not express MHC proteins. Activated microglia can express MHCII proteins and participate in antigen presentation.

MyD88: *Myeloid differentiation primary response protein 88*. Adapter protein that mediates signaling of the MyD88-dependent pathway in response to TLRs (All except TLR3) and IL-1β activation. Signaling of the MyD88-dependent pathway leads to activation of NF-κB and AP-1 transcription factors.

NAc: *Nucleus accumbens*. Brain region involved reward, motivation, and reinforcement. Further subdivided into the NAc core and shell. The NAc receives inputs from the PFC and amygdala and sends outputs ventral pallidum which projects to the PFC. The NAc is primarily composed of dopaminergic neurons.

NADPH: *Nicotinamide adenine dinucleotide phosphate*. NADPH oxidase generates superoxide anion by transferring electrons from NADPH and coupling them to oxygen. Superoxide anion is a reactive free-radical that can undergo further reactions to produce reactive oxygen species.

NF-κB: *Nuclear factor kappa-light-chain-enhancer of activated B cells*. Transcription factor containing 5 proteinsthat is activated in response to immune signaling including TLR signaling. Phosphorylation leads to activation and translocation to the nucleus where NF-κB transcribes cytokines and reactive oxygen species.

NO: *Nitric oxide*. NO is a free radical synthesized from oxygen and NAPDH by NOS enzymes. NO diffuses easily and acts as a

paracrine and autocrine signaling molecule. NO is generated by immune cells as part of the immune response and can also activate immune signaling. NO can modulate neurotransmission and promote apopotosis.

PBMC: *Peripheral blood mononuclear cell*. Peripheral blood cells that have a round nucleus, including T-cells, B-cells, NK cells, and monocytes. PBMCs respond to immune stimuli in the blood and respond by producing cytokines.

PFC: *Prefrontal Cortex*. Region of the frontal cortex that is responsible for executive functions like decision making, impulse inhibition, and planning. The PFC also integrates information from other brain regions, including those that regulate reward, anxiety, and memory.

PAMP: *Pathogen-associated molecular patterns*. Molecules that are associated with pathogens and can be recognized by pattern recognition receptors to mount an immune response. PAMPs include components of bacteria and viruses.

PRR: *Pattern recognition receptor*. Protein components of the innate immune system that recognize PAMPs and DAMPs. PRRs include Toll-like receptors (TLR), C-type lectin receptors (CLR), Nucleotide oliogmerisation receptors (NLR), and RIG-1 like receptors (RLR).

ROS: *Reactive oxygen species*. Chemically reactive species that contain oxygen, including peroxides, superoxide, hydroxyl radical, and singlet oxygen. Increased ROS levels can cause cellular damage and apoptosis.

Siglech: *Sialic acid binding Ig-like lectin H*. Member of the Siglec family: cell surface proteins that bind sialic acid and are primarily found on immune cells. Siglech is expressed on dendritic cells and microglia and is proposed to regulate microglial function under immune conditions. It also modulates the secretion of Type I interferons.

SMAD: *Mothers against decapentaplegic homolog*. Family of proteins that signal from TGF-β to the nucleus where they act as transcription factors. SMADs are broken into 3 classes, R-SMAD (SMAD1, SMAD2, SMAD3, SMAD5, and SMAD 8/9), co-SMADs (SMAD4), and I-SMADs (SMAD6, SMAD7).

TGF-β: *Transforming growth factor beta*. Cytokine that includes several isoforms (TGF-β 1-4). TGF-β proteins are synthesized as precursor molecules that can be activated by matrix metalloproteinases, pH, ROS, TGF-β, thrombospondin, and integrins. TGF-β complexes bind the TGF-β receptors leading to signaling through the SMAD pathway. TGF-β can also signal through a SMAD-independent apoptotic pathway.

TLR2: *Toll-like receptor 2*. Cell surface toll-like receptor that responds to glycolipids and lipoproteins on bacterial peptidoglycans and signals through the MyD88-dependent pathway.

TLR3: *Toll-like receptor 3*. Endosomal toll-like receptor that recognizes double stranded RNA and signals through the TRIF-dependent pathway. TLR3 is also activated in response to Poly I:C, a synthetic analog of double-stranded RNA.

TLR4: *Toll-like receptor 4*. Toll-like receptor that responds to lipopolysaccharide on gram negative bacteria. TLR4 can signal through both the MyD88-dependent and TRIF-dependent pathways. TLR4 can signal both from the cell membrane and from endosomes.

TLR7: *Toll-like receptor 7*. Endosomal toll-like receptor that recognizes single-stranded RNA. Although TLR7 signals through the MyD88-dependent pathway, it can activate both NF-κB and Type I interferons.

TNFα: *Tumor necrosis factor alpha*. Pro-inflammatory cytokine that is released in response to TLR signaling and NF-κB activation. TNF can bind to 2 receptors, TNFR1 and TNFR2. TNF binding to receptors can lead to NF-κB activation, AP-1 activation, and apoptosis.

TRAF3: *TNF receptor-associated factor 3*. TRAF3 is involved in signaling through the TRIF-dependent pathway.

TRAF6: *TNF receptor-associated factor 6*. TRAF6 is involved in signaling through the MyD88-dependent pathway.

TRIF: *TIR-domain-containing adapter-inducing interferon-β, also known as TICAM1*. TRIF is the adapter protein that mediates signaling from TLR3 and TLR4 leading to the activation of IRF3 and the production of type I interferons.

TSPO: *Translocator protein; also known as peripheral benzodiazepine receptor (PBR)*. Mitochondrial protein that is often using as a marker of microglial activation. Radioligands for TSPO are used for imaging studies as a way to measure microglial activation *in vivo*.

WGCNA: *Weighted gene co-expression network analysis*. Systems biology method for describing the correlation patterns among genes across gene expression data. WGCNA finds modules or clusters of highly correlated genes that can be related to external sample traits.

References

Abernathy, K., Chandler, L.J. & Woodward, J.J., 2010. Alcohol and the prefrontal cortex. *International review of neurobiology*, 91, pp.289–320.

Agrawal, R.G. et al., 2013. Bioinformatics Analyses Reveal Age-Specific Neuroimmune Modulation as a Target for Treatment of High Ethanol Drinking. *Alcoholism: Clinical and Experimental Research*, 38(2), pp.428–437.

Agrawal, R.G., Hewetson, A., George, C.M., Syapin, P.J. & Bergeson, S.E., 2011a. Minocycline reduces ethanol drinking. *Brain Behavior and Immunity*, 25 Suppl 1, pp.S165–9.

Agrawal, R.G., Hewetson, A., George, C.M., Syapin, P.J. & Bergeson, S.E., 2011b. Minocycline reduces ethanol drinking. *Brain Behavior and Immunity*, 25 Suppl 1, pp.S165–9.

Alfonso-Loeches, S. et al., 2010. Pivotal Role of TLR4 Receptors in Alcohol-Induced Neuroinflammation and Brain Damage. *Journal of Neuroscience*, 30(24), pp.8285–8295.

Alfonso-Loeches, S., Pascual, M. & Guerri, C., 2013. Gender differences in alcohol-induced neurotoxicity and brain damage. *Toxicology*, 311(1-2), pp.27–34.

American Psychiatric Association, 2013. *Diagnostic and statistical manual of mental disorders (5th ed.)*,

Anggayasti, W.L. et al., 2017. The self-association of HMGB1 and its possible role in the binding to DNA and cell membrane receptors. *FEBS letters*, 591(2), pp.282–294.

Anon, *Servier Medical Art*, Available at: http://servier.com/Powerpoint-image-bank.

Antón, M. et al., 2016. Oleoylethanolamide prevents neuroimmune HMGB1/TLR4/NF-kB danger signaling in rat frontal cortex and depressive-like behavior induced by ethanol binge administration. *Addiction Biology*, pp.n/a–n/a.

Asai, H. et al., 2015. Depletion of microglia and inhibition of exosome synthesis halt tau propagation. *Nature Neuroscience*, 18(11), pp.1584–1593.

Askew, K. et al., 2017. Coupled Proliferation and Apoptosis Maintain the Rapid Turnover of Microglia in the Adult Brain. *Cell Reports*, 18(2), pp.391–405.

Aurelian, L. et al., 2016. TLR4 signaling in VTA dopaminergic neurons regulates impulsivity through tyrosine hydroxylase modulation. *Translational psychiatry*, 6(5), p.e815.

Bajo, M. et al., 2014. Innate immune factors modulate ethanol interaction with GABAergic transmission in mouse central amygdala. *Brain Behavior and Immunity*, 40, pp.191–202.

Bajo, M., Herman, M.A., et al., 2015. Role of the IL-1 receptor antagonist in ethanol-induced regulation of GABAergic transmission in the central amygdala. *Brain Behavior and Immunity*, 45, pp.189–197.

Bajo, M., Varodayan, F.P., et al., 2015. IL-1 interacts with ethanol effects on GABAergic transmission in the mouse central amygdala. *Frontiers in pharmacology*, 6(19), p.49.

Bal-Price, A. & Brown, G.C., 2001. Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 21(17), pp.6480–6491.

Banks, W.A. & Robinson, S.M., 2010. Minimal penetration of lipopolysaccharide across the murine blood-brain barrier. *Brain Behavior and Immunity*, 24(1), pp.102–109.

Barnes, J., Mondelli, V. & Pariante, C.M., 2017. Genetic Contributions of Inflammation to Depression. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, 42(1), pp.81–98.

Bautista, A.P., 2002. Chronic alcohol intoxication primes Kupffer cells and endothelial cells for enhanced CC-chemokine production and concomitantly suppresses phagocytosis and chemotaxis. *Frontiers in bioscience : a journal and virtual library*, 7, pp.a117–25.

Bechara, A., 2005. Decision making, impulse control and loss of willpower to resist drugs: a neurocognitive perspective. *Nature Neuroscience*, 8(11), pp.1458–1463.

Bell-Temin, H. et al., 2013. Quantitative Proteomic Characterization of Ethanol-Responsive Pathways in Rat Microglial Cells. *Journal of Proteome Research*, 12(5), pp.2067–2077.

Bennett, M.L. et al., 2016. New tools for studying microglia in the mouse and human CNS. *Proceedings of the National Academy of Sciences of the United States of America*, 113(12), pp.E1738–46.

Bernhardi, von, R. et al., 2015. Role of TGFβ signaling in the pathogenesis of

Alzheimer's disease. *Frontiers in cellular neuroscience*, 9, p.426.

Béchade, C. et al., 2014. NOS2 expression is restricted to neurons in the healthy brain but is triggered in microglia upon inflammation. *Glia*, 62(6), pp.956–963.

Blanco, A.M. et al., 2008. Ethanol mimics ligand-mediated activation and endocytosis of IL-1RITLR4 receptors via lipid raftscaveolae in astroglial cells. *Journal of Neurochemistry*, 106(2), pp.625–639.

Blanco, A.M. et al., 2005. Involvement of TLR4/type I IL-1 receptor signaling in the induction of inflammatory mediators and cell death induced by ethanol in cultured astrocytes. *The Journal of Immunology*, 175(10), pp.6893–6899.

Blasius, A.L. et al., 2006. Siglec-H is an IPC-specific receptor that modulates type I IFN secretion through DAP12. *Blood*, 107(6), pp.2474–2476.

BLEDNOV, Y. et al., 2005. Perturbation of chemokine networks by gene deletion alters the reinforcing actions of ethanol. *Behavioural Brain Research*, 165(1), pp.110–125.

Blednov, Y.A. et al., 2017. Ethanol Consumption in Mice Lacking CD14, TLR2, TLR4, or MyD88. *Alcoholism: Clinical and Experimental Research*.

Blednov, Y.A., Benavidez, J.M., et al., 2011. Activation of inflammatory signaling by lipopolysaccharide produces a prolonged increase of voluntary alcohol intake in mice. *Brain Behavior and Immunity*, 25, pp.S92–S105.

Blednov, Y.A., Ponomarev, I., et al., 2011. Neuroimmune regulation of alcohol consumption: behavioral validation of genes obtained from genomic studies. *Addiction Biology*, 17(1), pp.108–120.

Boche, D. et al., 2003. Transforming growth factor-beta 1-mediated neuroprotection against excitotoxic injury in vivo. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, 23(10), pp.1174–1182.

Boettiger, C.A. et al., 2007. Immediate reward bias in humans: fronto-parietal networks and a role for the catechol-O-methyltransferase 158(Val/Val) genotype. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 27(52), pp.14383–14391.

Boettiger, C.A. et al., 2009. Now or Later? An fMRI study of the effects of endogenous opioid blockade on a decision-making network. *Pharmacology, Biochemistry and Behavior*, 93(3), pp.291–299.

Bongarzone, E.R. et al., 2000. Platelet-derived growth factor and basic fibroblast growth

factor regulate cell proliferation and the expression of notch-1 receptor in a new oligodendrocyte cell line. *Journal of neuroscience research*, 62(3), pp.319–328.

Borysiewicz, E. et al., 2013. TLR3 ligation protects human astrocytes against oxidative stress. *Journal of neuroimmunology*, 255(1-2), pp.54–59.

Bossù, P. et al., 2012. A single intraperitoneal injection of endotoxin in rats induces long-lasting modifications in behavior and brain protein levels of TNF-α and IL-18. *???*, 9(1), pp.1–1.

Bouchery, E.E. et al., 2011. Economic costs of excessive alcohol consumption in the U.S., 2006. *American journal of preventive medicine*, 41(5), pp.516–524.

Breese, G.R. et al., 2008. Repeated lipopolysaccharide (LPS) or cytokine treatments sensitize ethanol withdrawal-induced anxiety-like behavior. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, 33(4), pp.867–876.

Bsibsi, M. et al., 2002. Broad expression of Toll-like receptors in the human central nervous system. *Journal of neuropathology and experimental neurology*, 61(11), pp.1013–1021.

Bustin, S.A. et al., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical chemistry*, 55(4), pp.611–622.

Butovsky, O. et al., 2013. Identification of a unique TGF-β–dependent molecular and functional signature in microglia. *Nature Neuroscience*, 17(1), pp.131–143.

Cahoy, J.D. et al., 2008. A Transcriptome Database for Astrocytes, Neurons, and Oligodendrocytes: A New Resource for Understanding Brain Development and Function. *Journal of Neuroscience*, 28(1), pp.264–278.

Carniglia, L. et al., 2017. Neuropeptides and Microglial Activation in Inflammation, Pain, and Neurodegenerative Diseases. *Mediators of inflammation*, 2017(6), pp.5048616–23.

Carpenter, S. et al., 2014. Post-transcriptional regulation of gene expression in innate immunity. *Nature Reviews Immunology*, 14(6), pp.361–376.

Center for Behavioral Health Statistics and Quality, Substance Abuse and Mental Health Services AdministrationU.S. Department of Health and Human Services, *Results from the 2015 National Survey on Drug Use and Health*, Available at: https://www.samhsa.gov/data/sites/default/files/NSDUH-DetTabs-2015/NSDUH-DetTabs-2015/NSDUH-DetTabs-2015.htm#tab5-6b.

Centers for Disease Control and Prevention (CDC), *Alcohol and Public Health: Alcohol-Related Disease Impact (ARDI)*, Available at: https://nccd.cdc.gov/DPH_ARDI/Default/Report.aspx?T=AAM&P=f6d7eda7-036e-4553-9968-9b17ffad620e&R=d7a9b303-48e9-4440-bf47-070a4827e1fd&M=8E1C5233-5640-4EE8-9247-1ECA7DA325B9&F=&D=.

Cha, A. et al., 2012. Antilipogenic and anti-inflammatory activities of Codonopsis lanceolata in mice hepatic tissues after chronic ethanol feeding. *Journal of biomedicine & biotechnology*, 2012(30), pp.141395–13.

Chen, C.P. et al., 2006. Ethanol induces apoptotic death of developing beta-endorphin neurons via suppression of cyclic adenosine monophosphate production and activation of transforming growth factor-beta1-linked apoptotic signaling. *Molecular Pharmacology*, 69(3), pp.706–717.

Chen, E.Y. et al., 2013. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC bioinformatics*, 14(1), p.128.

Cherry, J.D., Olschowka, J.A. & Banion, M.K.O., 2014. Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. 11(1), pp.1–15.

Chiu, I.M. et al., 2013. A Neurodegeneration-Specific Gene-Expression Signature of Acutely Isolated Microglia from an Amyotrophic Lateral Sclerosis Mouse Model. *Cell Reports*, 4(2), pp.385–401.

Coleman, L.G., Zou, J. & Crews, F.T., 2017. Microglial-derived miRNA let-7 and HMGB1 contribute to ethanol-induced neurotoxicity via TLR7. *Journal of Neuroinflammation*, 14(1), p.22.

Colton, C.A., 2009. Heterogeneity of microglial activation in the innate immune response in the brain. *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology*, 4(4), pp.399–418.

Conner, C. et al., 2014. Repressing notch signaling and expressing TNFα are sufficient to mimic retinal regeneration by inducing Müller glial proliferation to generate committed progenitor cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 34(43), pp.14403–14419.

Crabbe, J.C. et al., 2012. Intermittent Availability of Ethanol Does Not Always Lead to Elevated Drinking in Mice. *Alcohol and Alcoholism*, 47(5), pp.509–517.

Crews, F. et al., 2006. BHT Blocks NF-?B activation and Ethanol-Induced Brain Damage. *Alcoholism: Clinical and Experimental Research*, 30(11), pp.1938–1949.

Crews, F.T., 2012. Immune function genes, genetics, and the neurobiology of addiction. *Alcohol research : current reviews*, 34(3), pp.355–361.

Crews, F.T. & Boettiger, C.A., 2009a. Impulsivity, frontal lobes and risk for addiction. *Pharmacology, Biochemistry and Behavior*, 93(3), pp.237–247.

Crews, F.T. & Boettiger, C.A., 2009b. Impulsivity, frontal lobes and risk for addiction. *Pharmacology, Biochemistry and Behavior*, 93(3), pp.237–247.

Crews, F.T. & Nixon, Kim, 2009. Mechanisms of neurodegeneration and regeneration in alcoholism. *Alcohol and alcoholism (Oxford, Oxfordshire)*, 44(2), pp.115–127.

Crews, F.T. & Vetreno, R.P., 2016. Mechanisms of neuroimmune gene induction in alcoholism. *Psychopharmacology*, 233(9), pp.1543–1557.

Crews, F.T. et al., 2013. High Mobility Group Box 1/Toll-like Receptor Danger Signaling Increases Brain Neuroimmune Activation in Alcohol Dependence. *Biological Psychiatry*, 73(7), pp.602–612.

Crews, F.T. et al., 2015. Neuroimmune Function and the Consequences of Alcohol Exposure. *Alcohol research : current reviews*, 37(2), pp.331–41– 344–51.

Crews, F.T. et al., 2017. Toll-like receptor signaling and stages of addiction. *Psychopharmacology*, 108(Suppl 1), pp.218–16.

Crews, F.T., Zou, J. & Qin, L., 2011. Induction of innate immune genes in brain create the neurobiology of addiction. *Brain Behavior and Immunity*, 25(S1), pp.S4–S12.

Cui, C., Shurtleff, D. & Harris, R.A., 2014. Neuroimmune mechanisms of alcohol and drug addiction. *International review of neurobiology*, 118, pp.1–12.

Dantzer, R. et al., 2008. From inflammation to sickness and depression: when the immune system subjugates the brain. *Nature Reviews Neuroscience*, 9(1), pp.46–56.

Darmanis, S. et al., 2015. A survey of human brain transcriptome diversity at the single cell level. *Proceedings of the National Academy of Sciences*, 112(23), pp.7285–7290.

Datto, M.B. et al., 1999. Targeted disruption of Smad3 reveals an essential role in transforming growth factor beta-mediated signal transduction. *Molecular and cellular biology*, 19(4), pp.2495–2504.

Davis, R.L. & Syapin, P.J., 2004. Chronic ethanol inhibits CXC chemokine ligand 10 production in human A172 astroglia and astroglial-mediated leukocyte chemotaxis. *Neuroscience letters*, 362(3), pp.220–225.

de Haas, A.H., Boddeke, H.W.G.M. & Biber, K., 2008. Region-specific expression of immunoregulatory proteins on microglia in the healthy CNS. *Glia*, 56(8), pp.888–894.

De Jager, P.L. et al., 2014. Alzheimer's disease: early alterations in brain DNA methylation at ANK1, BIN1, RHBDF2 and other loci. *Nature Neuroscience*, 17(9), pp.1156–1163.

Dennis, C.V. et al., 2014. Microglial proliferation in the brain of chronic alcoholics with hepatic encephalopathy. *Metabolic brain disease*, 29(4), pp.1027–1039.

Diamond, I. & Gordon, A.S., 1997. Cellular and molecular neuroscience of alcoholism. *Physiological reviews*, 77(1), pp.1–20.

Dobin, A. et al., 2013. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics (Oxford, England)*, 29(1), pp.15–21.

Dobolyi, A. et al., 2012. The neuroprotective functions of transforming growth factor beta proteins. *International journal of molecular sciences*, 13(7), pp.8219–8258.

Dooley, S. & Dijke, ten, P., 2012. TGF-β in progression of liver disease. *Cell and tissue research*, 347(1), pp.245–256.

Dou, X. et al., 2014. [Down-regulation of TSPO expression doesn't affect the productions of TNF-α, IL-1β and IL-6 in LPS-stimulated BV-2 microglia]. *Xi bao yu fen zi mian yi xue za zhi = Chinese journal of cellular and molecular immunology*, 30(9), pp.897–900.

Edwards, S. et al., 2011. Current and promising pharmacotherapies, and novel research target areas in the treatment of alcohol dependence: a review. *Current pharmaceutical design*, 17(14), pp.1323–1332.

Ewald, S.E. & Barton, G.M., 2011. Nucleic acid sensing Toll-like receptors in autoimmunity. *Current opinion in immunology*, 23(1), pp.3–9.

Faraco, G. et al., 2007. High mobility group box 1 protein is released by neural cells upon different stresses and worsens ischemic neurodegeneration in vitro and in vivo. *Journal of Neurochemistry*, 103(2), pp.590–603.

Farris, S.P. et al., 2014. Transcriptome organization for chronic alcohol abuse in human brain. *Molecular Psychiatry*, 20(11), pp.1438–1447.

Fernandez-Lizarbe, S. et al., 2008. Lipid rafts regulate ethanol-induced activation of TLR4 signaling in murine macrophages. *Molecular Immunology*, 45(7), pp.2007–2016.

Fernandez-Lizarbe, S., Montesinos, J. & Guerri, C., 2013. Ethanol induces TLR4/TLR2 association, triggering an inflammatory response in microglial cells. *Journal of Neurochemistry*, 126(2), pp.261–273.

Fernandez-Lizarbe, S., Pascual, M. & Guerri, C., 2009. Critical Role of TLR4 Response in the Activation of Microglia Induced by Ethanol. *The Journal of Immunology*, 183(7), pp.4733–4744.

Ferrier, L. et al., 2006. Impairment of the intestinal barrier by ethanol involves enteric microflora and mast cell activation in rodents. *The American journal of pathology*, 168(4), pp.1148–1154.

Galic, M.A., Riazi, K. & Pittman, Q.J., 2012. Cytokines and brain excitability. *Frontiers in neuroendocrinology*, 33(1), pp.116–125.

Gallucci, R.M. & Meadows, G.G., 1996. Ethanol consumption suppresses the IL2-induced proliferation of NK cells. *Toxicology and applied pharmacology*, 138(1), pp.90–97.

Galvez-Contreras, A.Y. et al., 2016. Growth factors as clinical biomarkers of prognosis and diagnosis in psychiatric disorders. *Cytokine & growth factor reviews*, 32, pp.85–96.

Gambuzza, M.E. et al., 2014. Toll-like receptors in Alzheimer's disease: a therapeutic perspective. *CNS & neurological disorders drug targets*, 13(9), pp.1542–1558.

Gao, F. et al., 2014. Acute lipopolysaccharide exposure facilitates epileptiform activity via enhanced excitatory synaptic transmission and neuronal excitability in vitro. *Neuropsychiatric disease and treatment*, 10, pp.1489–1495.

García Bueno, B. et al., 2016. Innate immune receptor Toll-like receptor 4 signalling in neuropsychiatric diseases. *Neuroscience and biobehavioral reviews*, 64, pp.134–147.

Gaudet, A.D. & Popovich, P.G., 2014. Extracellular matrix regulation of inflammation in the healthy and injured spinal cord. *Experimental Neurology*, 258, pp.24–34.

Gąsiorowski, K. et al., 2017. RAGE-TLR Crosstalk Sustains Chronic Inflammation in Neurodegeneration. *Molecular neurobiology*, 12(5), p.593.

Gemma, C. & Bachstetter, A.D., 2013. The role of microglia in adult hippocampal neurogenesis. *Frontiers in cellular neuroscience*, 7, p.229.

George, J. et al., 2016. Microglia-derived purines modulate mossy fibre synaptic transmission and plasticity through P2X4 and A1 receptors. G. Maccaferri, ed. *European Journal of Neuroscience*, 43(10), pp.1366–1378.

Gesuete, R., Kohama, S.G. & Stenzel-Poore, M.P., 2014. Toll-like receptors and ischemic brain injury. *Journal of neuropathology and experimental neurology*, 73(5), pp.378–386.

Gonzalez-Pena, D. et al., 2016. Microglia Transcriptome Changes in a Model of Depressive Behavior after Immune Challenge S. K. Ahuja, ed. *PLoS ONE*, 11(3), pp.e0150858–28.

Goral, J., Karavitis, J. & Kovacs, E.J., 2008. Exposure-dependent effects of ethanol on the innate immune system. *Alcohol (Fayetteville, N.Y.)*, 42(4), pp.237–247.

Gorina, R. et al., 2010. Astrocyte TLR4 activation induces a proinflammatory environment through the interplay between MyD88-dependent NFκB signaling, MAPK, and Jak1/Stat1 pathways. *Glia*, 59(2), pp.242–255.

Grabert, K. et al., 2016. Microglial brain region-dependent diversity and selective regional sensitivities to aging. *Nature Neuroscience*, 19(3), pp.504–516.

Grace, P.M. et al., 2014. Activation of adult rat CNS endothelial cells by opioid-induced toll-like receptor 4 (TLR4) signaling induces proinflammatory, biochemical, morphological, and behavioral sequelae. *NEUROSCIENCE*, 280, pp.299–317.

Grant, B.F. et al., 2015. Epidemiology of DSM-5 Alcohol Use Disorder: Results From the National Epidemiologic Survey on Alcohol and Related Conditions III. *JAMA psychiatry*, 72(8), pp.757–766.

Grootjans, J. et al., 2016. The unfolded protein response in immunity and inflammation. *Nature Reviews Immunology*, 16(8), pp.469–484.

Gruber, A.J. et al., 2010. More is less: a disinhibited prefrontal cortex impairs cognitive flexibility. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 30(50), pp.17102–17110.

Gruol, D.L., 2016. Impact of Increased Astrocyte Expression of IL-6, CCL2 or CXCL10 in Transgenic Mice on Hippocampal Synaptic Function. *Brain sciences*, 6(2), p.19.

Guadagno, J. et al., 2013. Microglia-derived TNFα induces apoptosis in neural precursor cells via transcriptional activation of the Bcl-2 family member Puma. *Cell death & disease*, 4(3), p.e538.

Guo, J.U. et al., 2011. Neuronal activity modifies the DNA methylation landscape in the adult brain. *Nature Neuroscience*, 14(10), pp.1345–1351.

Guo, X. & Wang, X.-F., 2009. Signaling cross-talk between TGF-beta/BMP and other pathways. *Cell research*, 19(1), pp.71–88.

Hanke, M.L. & Kielian, T., 2011. Toll-like receptors in health and disease in the brain: mechanisms and therapeutic potential. *Clinical science (London, England : 1979)*, 121(9), pp.367–387.

Harper, C., 2009. The Neuropathology of Alcohol-Related Brain Damage. *Alcohol and Alcoholism*, 44(2), pp.136–140.

Harris, R.A. et al., 2017. Genetic and Pharmacologic Manipulation of TLR4 Has Minimal Impact on Ethanol Consumption in Rodents. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 37(5), pp.1139–1155.

Harry, G.J., 2013. Microglia during development and aging. *Pharmacology and Therapeutics*, 139(3), pp.313–326.

Hasan, M. & Yan, N., 2016. Therapeutic potential of targeting TBK1 in autoimmune diseases and interferonopathies. *Pharmacological Research*, 111, pp.336–342.

Hayley, S., 2014. The neuroimmune-neuroplasticity interface and brain pathology. *Frontiers in cellular neuroscience*, 8(68), p.419.

He, J. & Crews, F.T., 2008. Increased MCP-1 and microglia in various regions of the human alcoholic brain. *Experimental Neurology*, 210(2), pp.349–358.

Heneka, M.T., Kummer, M.P. & Latz, E., 2014. Innate immune activation in neurodegenerative disease. *Nature Reviews Immunology*, 14(7), pp.463–477.

Hess, J., Angel, P. & Schorpp-Kistner, M., 2004. AP-1 subunits: quarrel and harmony among siblings. *Journal of cell science*, 117(Pt 25), pp.5965–5973.

Hickman, S.E. et al., 2013. The microglial sensome revealed by direct RNA sequencing. *Nature Neuroscience*, 16(12), pp.1896–1905.

Hillmer, A.T. et al., 2017. In vivo imaging of translocator protein, a marker of activated microglia, in alcohol dependence. *Molecular Psychiatry*.

Hingson, R. et al., 2005. Magnitude of alcohol-related mortality and morbidity among U.S. college students ages 18-24: changes from 1998 to 2001. *Annual review of public health*, 26(1), pp.259–279.

Hirotani, T. et al., 2005. Regulation of lipopolysaccharide-inducible genes by MyD88 and Toll/IL-1 domain containing adaptor inducing IFN-beta. *Biochemical and Biophysical Research Communications*, 328(2), pp.383–392.

Hocevar, B.A. & Howe, P.H., 1998. Mechanisms of TGF-beta-induced cell cycle arrest. *Mineral and electrolyte metabolism*, 24(2-3), pp.131–135.

Hope, B.T. et al., 1994. Induction of a long-lasting AP-1 complex composed of altered Fos-like proteins in brain by chronic cocaine and other chronic treatments. *Neuron*, 13(5), pp.1235–1244.

Hou, B., Reizis, B. & DeFranco, A.L., 2008. Toll-like Receptors Activate Innate and Adaptive Immunity by using Dendritic Cell-Intrinsic and -Extrinsic Mechanisms. *Immunity*, 29(2), pp.272–282.

Hsu, H. et al., 1996. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell*, 84(2), pp.299–308.

Hu, X. et al., 2014. Neurobiology of microglial action in CNS injuries: receptor-mediated signaling mechanisms and functional roles. *Progress in neurobiology*, 119-120, pp.60–84.

Hurle, M.R. et al., 2013. Computational drug repositioning: from data to therapeutics. *Clinical pharmacology and therapeutics*, 93(4), pp.335–341.

Imai, Y. & Kohsaka, S., 2002. Intracellular signaling in M-CSF-induced microglia activation: role of Iba1. *Glia*, 40(2), pp.164–174.

Jack, C.S. et al., 2005. TLR signaling tailors innate immune responses in human microglia and astrocytes. *The Journal of Immunology*, 175(7), pp.4320–4330.

Janot, L. et al., 2008. CD14 works with toll-like receptor 2 to contribute to recognition and control of Listeria monocytogenes infection. *The Journal of infectious diseases*, 198(1), pp.115–124.

Ju, L. et al., 2015. Dual polarization of microglia isolated from mixed glial cell cultures. *Journal of neuroscience research*, 93(9), pp.1345–1352.

June, H.L. et al., 2015. CRF-Amplified Neuronal TLR4/MCP-1 Signaling Regulates Alcohol Self-Administration. 40(6), pp.1549–1559.

Kalk, N.J. et al., 2017. Decreased hippocampal translocator protein (18 kDa) expression in alcohol dependence: a [(11)C]PBR28 PET study. *Translational psychiatry*, 7(1), p.e996.

Kamata, H. et al., 2005. Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell*, 120(5), pp.649–661.

Kane, C.J.M. et al., 2013. Effects of Ethanol on Immune Response in the Brain: Region-Specific Changes in Adolescent Versus Adult Mice. *Alcoholism: Clinical and*

Experimental Research, 38(2), pp.384–391.

Kaneko, Y.S. et al., 2015. Lipopolysaccharide treatment arrests the cell cycle of BV-2 microglial cells in G_1 phase and protects them from UV light-induced apoptosis. *Journal of neural transmission (Vienna, Austria : 1996)*, 122(2), pp.187–199.

Kawai, T. & Akira, S., 2007. Signaling to NF-kappaB by Toll-like receptors. *Trends in molecular medicine*, 13(11), pp.460–469.

Kawai, T. & Akira, S., 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature Immunology*, 11(5), pp.373–384.

Kawasaki, T. & Kawai, T., 2014. Toll-like receptor signaling pathways. *Frontiers in immunology*, 5, p.461.

Kerns, R.T. et al., 2005. Ethanol-responsive brain region expression networks: implications for behavioral responses to acute ethanol in DBA/2J versus C57BL/6J mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 25(9), pp.2255–2266.

Keshavarzian, A. et al., 1999. Leaky gut in alcoholic cirrhosis: a possible mechanism for alcohol-induced liver damage. *The American journal of gastroenterology*, 94(1), pp.200–207.

Kielian, T., 2006. Toll-like receptors in central nervous system glial inflammation and homeostasis. *Journal of neuroscience research*, 83(5), pp.711–730.

Kim, Y.-K. et al., 2009. Increased transforming growth factor-beta1 in alcohol dependence. *Journal of Korean medical science*, 24(5), pp.941–944.

Knapp, D.J. et al., 2016. Stress and Withdrawal from Chronic Ethanol Induce Selective Changes in Neuroimmune mRNAs in Differing Brain Sites. *Brain sciences*, 6(3), p.25.

Koeglsperger, T., Li, S., Brenneis, C., Saulnier, J.L., Mayo, L., Carrier, Y., Selkoe, D.J. & Weiner, H.L., 2013a. Impaired glutamate recycling and GluN2B-mediated neuronal calcium overload in mice lacking TGF-β1 in the CNS. *Glia*, 61(6), pp.985–1002.

Koeglsperger, T., Li, S., Brenneis, C., Saulnier, J.L., Mayo, L., Carrier, Y., Selkoe, D.J. & Weiner, H.L., 2013b. Impaired glutamate recycling and GluN2B-mediated neuronal calcium overload in mice lacking TGF-β1 in the CNS. *Glia*, 61(6), pp.985–1002.

Kondo, S., Kohsaka, S. & Okabe, S., 2011. Long-term changes of spine dynamics and microglia after transient peripheral immune response triggered by LPS in vivo. *Molecular brain*, 4(1), p.27.

Konsoula, Z. & Barile, F.A., 2012. Epigenetic histone acetylation and deacetylation mechanisms in experimental models of neurodegenerative disorders. *Journal of pharmacological and toxicological methods*, 66(3), pp.215–220.

Koob, G.F. & Volkow, N.D., 2010. Neurocircuitry of addiction. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, 35(1), pp.217–238.

Koob, G.F., Arends, M.A. & Le Moal, M., 2014. *Drugs, Addiction, and the Brain*, San Diego: Elsevier.

Kopatz, J. et al., 2013. Siglec-h on activated microglia for recognition and engulfment of glioma cells. *Glia*, 61(7), pp.1122–1133.

Kovács, K.J., 2017. Microglia and drug-induced plasticity in reward-related neuronal circuits. pp.1–3.

Kraft, A.D., McPherson, C.A. & Harry, G.J., 2009. Heterogeneity of microglia and TNF signaling as determinants for neuronal death or survival. *Neurotoxicology*, 30(5), pp.785–793.

Kuleshov, M.V. et al., 2016. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic acids research*, 44(W1), pp.W90–7.

Lacagnina, M.J., Rivera, P.D. & Bilbo, S.D., 2016. Glial and Neuroimmune Mechanisms as Critical Modulators of Drug Use and Abuse. pp.1–22.

Lamb, J. et al., 2006. The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. *Science*, 313(5795), pp.1929–1935.

Langfelder, P. & Horvath, S., 2008. WGCNA: an R package for weighted correlation network analysis. *BMC bioinformatics*, 9(1), p.559.

Lasek, A.W., 2016. Effects of Ethanol on Brain Extracellular Matrix: Implications for Alcohol Use Disorder. *Alcoholism: Clinical and Experimental Research*, 40(10), pp.2030–2042.

Lathia, J.D. et al., 2008. Toll-like receptor 3 is a negative regulator of embryonic neural progenitor cell proliferation. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 28(51), pp.13978–13984.

Lawrimore, C.J. & Crews, F.T., 2017. Ethanol, TLR3, and TLR4 agonists have unique innate immune responses in neuron-like SH-SY5Y and microglia-like BV2. *Alcoholism: Clinical and Experimental Research*.

Lawson, L.J. et al., 1990. Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. *NEUROSCIENCE*, 39(1), pp.151–170.

Leclercq, S. et al., 2012. Role of intestinal permeability and inflammation in the biological and behavioral control of alcohol-dependent subjects. *Brain Behavior and Immunity*, 26(6), pp.911–918.

Leclercq, S. et al., 2017. The link between inflammation, bugs, the intestine and the brain in alcohol dependence. *Translational psychiatry*, 7(2), p.e1048.

Leclercq, S., De Saeger, C., et al., 2014. Role of Inflammatory Pathways, Blood Mononuclear Cells, and Gut-Derived Bacterial Products in Alcohol Dependence. *Biological Psychiatry*, 76(9), pp.725–733.

Leclercq, S., Matamoros, S., et al., 2014. Intestinal permeability, gut-bacterial dysbiosis, and behavioral markers of alcohol-dependence severity. *Proceedings of the National Academy of Sciences of the United States of America*, 111(42), pp.E4485–93.

Lee, H.Y. et al., 2012. Changes of ribosomal protein S3 immunoreactivity and its new expression in microglia in the mice hippocampus after lipopolysaccharide treatment. *Cellular and molecular neurobiology*, 32(4), pp.577–586.

Lehnardt, S., 2009. Innate immunity and neuroinflammation in the CNS: The role of microglia in Toll-like receptor-mediated neuronal injury. *Glia*, pp.NA–NA.

Lehnardt, S. et al., 2002. The toll-like receptor TLR4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the CNS. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 22(7), pp.2478–2486.

Leow-Dyke, S. et al., 2012. Neuronal toll-like receptor 4 signaling induces brain endothelial activation and neutrophil transmigration in vitro. *Journal of Neuroinflammation*, 9(1), pp.1–1.

Lewohl, J.M. et al., 2005. Expression of MBP, PLP, MAG, CNP, and GFAP in the Human Alcoholic Brain. *Alcoholism: Clinical and Experimental Research*, 29(9), pp.1698–1705.

Lewohl, J.M., Wang, L., Miles, M.F., Zhang, L., Dodd, P.R. & Harris, R.A., 2000a. Gene expression in human alcoholism: microarray analysis of frontal cortex. *Alcoholism: Clinical and Experimental Research*, 24(12), pp.1873–1882.

Lewohl, J.M., Wang, L., Miles, M.F., Zhang, L., Dodd, P.R. & Harris, R.A., 2000b. Gene expression in human alcoholism: microarray analysis of frontal cortex. *Alcoholism: Clinical and Experimental Research*, 24(12), pp.1873–1882.

Li, M.D. et al., 2015. Aging-like changes in the transcriptome of irradiated microglia. *Glia*, 63(5), pp.754–767.

Li, Y. et al., 2012. Reciprocal regulation between resting microglial dynamics and neuronal activity in vivo. *Developmental cell*, 23(6), pp.1189–1202.

Liddelow, S.A. et al., 2017. Neurotoxic reactive astrocytes are induced by activated microglia. *Nature*, 541(7638), pp.481–487.

Lin, R. et al., 2015. TSPO, a Mitochondrial Outer Membrane Protein, Controls Ethanol-Related Behaviors in Drosophila. B. Lu, ed. *PLoS genetics*, 11(8), p.e1005366.

Lippai, D. et al., 2013. Alcohol-induced IL-1 in the brain is mediated by NLRP3/ASC inflammasome activation that amplifies neuroinflammation. *Journal of Leukocyte Biology*, 94(1), pp.171–182.

Listwak, S.J., Rathore, P. & Herkenham, M., 2013. Minimal NF-Î°B activity in neurons. *NEUROSCIENCE*, 250(C), pp.282–299.

Liu, Jianwen et al., 2006. Patterns of gene expression in the frontal cortex discriminate alcoholic from nonalcoholic individuals. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, 31(7), pp.1574–1582.

Liu, Juan et al., 2011. Binge alcohol drinking is associated with GABAA alpha2-regulated Toll-like receptor 4 (TLR4) expression in the central amygdala. *Proceedings of the National Academy of Sciences of the United States of America*, 108(11), pp.4465–4470.

Liu, Mingli et al., 2011. CXCL10/IP-10 in infectious diseases pathogenesis and potential therapeutic implications. *Cytokine & growth factor reviews*, 22(3), pp.121–130.

Liu, Xing-Jun et al., 2016. TLR signaling adaptor protein MyD88 in primary sensory neurons contributes to persistent inflammatory and neuropathic pain and neuroinflammation. *Nature Publishing Group*, 6, p.28188.

Lively, S. & Schlichter, L.C., 2013. The microglial activation state regulates migration and roles of matrix-dissolving enzymes for invasion. *Journal of Neuroinflammation*, 10(1), p.75.

Lodge, P.A. & Sriram, S., 1996. Regulation of microglial activation by TGF-beta, IL-10,

and CSF-1. *Journal of Leukocyte Biology*, 60(4), pp.502–508.

Love, M.I., Huber, W. & Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology*, 15(12), p.550.

Lovinger, D.M. & Crabbe, J.C., 2005. Laboratory models of alcoholism: treatment target identification and insight into mechanisms. *Nature Neuroscience*, 8(11), pp.1471–1480.

LUNDQUIST, F. et al., 1959. The metabolism of acetaldehyde in mammalian tissues: reactions in rat-liver suspensions under anaerobic conditions. *The Biochemical journal*, 72(3), pp.409–419.

Luo, X.-G. & Chen, S.-D., 2012. The changing phenotype of microglia from homeostasis to disease. *Translational neurodegeneration*, 1(1), p.9.

Maggio, N. et al., 2013. Prolonged systemic inflammation persistently modifies synaptic plasticity in the hippocampus: modulation by the stress hormones. *Frontiers in molecular neuroscience*, 6, p.46.

Marinelli, C. et al., 2015. Ligand engagement of Toll-like receptors regulates their expression in cortical microglia and astrocytes. *Journal of Neuroinflammation*, 12(1), p.244.

May, P.A. et al., 2009. Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. B. Paley, ed. *Developmental disabilities research reviews*, 15(3), pp.176–192.

Mayfield, J., Ferguson, L. & Harris, R.A., 2013. Neuroimmune signaling: a key component of alcohol abuse. *Current Opinion in Neurobiology*, pp.1–8.

Maze, I. et al., 2015. Critical Role of Histone Turnover in Neuronal Transcription and Plasticity. *Neuron*, 87(1), pp.77–94.

McCool, B.A., 2011. Ethanol modulation of synaptic plasticity. *Neuropharmacology*, 61(7), pp.1097–1108.

Medzhitov, R. & Horng, T., 2009. Transcriptional control of the inflammatory response. *Nature Reviews Immunology*, 9(10), pp.692–703.

Melendez, R.I., 2011. Intermittent (every-other-day) drinking induces rapid escalation of ethanol intake and preference in adolescent and adult C57BL/6J mice. *Alcoholism: Clinical and Experimental Research*, 35(4), pp.652–658.

Melvin, N.R. & Sutherland, R.J., 2010. Quantitative caveats of standard

immunohistochemical procedures: implications for optical disector-based designs. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*, 58(7), pp.577–584.

Miller, A.H. et al., 2013. Cytokine targets in the brain: impact on neurotransmitters and neurocircuits. *Depression and anxiety*, 30(4), pp.297–306.

Miller, E.K., Freedman, D.J. & Wallis, J.D., 2002. The prefrontal cortex: categories, concepts and cognition. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 357(1424), pp.1123–1136.

Milner, R. & Campbell, I.L., 2003. The extracellular matrix and cytokines regulate microglial integrin expression and activation. *The Journal of Immunology*, 170(7), pp.3850–3858.

Ming, Z. et al., 2015. Systemic lipopolysaccharide-mediated alteration of cortical neuromodulation involves increases in monoamine oxidase-A and acetylcholinesterase activity. *Journal of Neuroinflammation*, 12(1), p.37.

Miron, V.E., 2017. Microglia-driven regulation of oligodendrocyte lineage cells, myelination, and remyelination. *Journal of Leukocyte Biology*, pp.jlb.3RI1116–494R.

Mizee, M.R. et al., 2017. Isolation of primary microglia from the human post-mortem brain: effects of ante- and post-mortem variables. *Acta neuropathologica communications*, 5(1), p.16.

Mogensen, T.H., 2009. Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses. *Clinical Microbiology Reviews*, 22(2), pp.240–273.

Moriguchi, S. et al., 2007. Effects of ethanol on excitatory and inhibitory synaptic transmission in rat cortical neurons. *Alcoholism: Clinical and Experimental Research*, 31(1), pp.89–99.

Most, D. et al., 2015. The synaptoneurosome transcriptome: a model for profiling the emolecular effects of alcohol. *The Pharmacogenomics Journal*, 15(2), pp.177–188.

Most, D., Ferguson, L. & Harris, R.A., 2014. Molecular basis of alcoholism. *Handbook of clinical neurology*, 125, pp.89–111.

Most, D., Workman, E. & Harris, R.A., 2014. Synaptic adaptations by alcohol and drugs of abuse: changes in microRNA expression and mRNA regulation. *Frontiers in molecular neuroscience*, 7(e82565), p.85.

Mukherjee, P. et al., 2015. SARM1, Not MyD88, Mediates TLR7/TLR9-Induced Apoptosis in Neurons. *Journal of immunology (Baltimore, Md. : 1950)*, 195(10),

pp.4913–4921.

Mukherjee, S. et al., 2008. Consequences of alcohol consumption on neurotransmitters - an overview. *Current neurovascular research*, 5(4), pp.266–272.

Mulligan, M.K. et al., 2006. Toward understanding the genetics of alcohol drinking through transcriptome meta-analysis. *Proceedings of the National Academy of Sciences*, 103(16), pp.6368–6373.

Murphy-Royal, C. et al., 2017. Astroglial glutamate transporters in the brain: Regulating neurotransmitter homeostasis and synaptic transmission. *Journal of neuroscience research*, 5(Pt 2), p.ra27.

Nakagawa, Y. & Chiba, K., 2014. Role of microglial m1/m2 polarization in relapse and remission of psychiatric disorders and diseases. *Pharmaceuticals (Basel, Switzerland)*, 7(12), pp.1028–1048.

National Center for Statistics and Analysis, 2015. 2014 Crash Data Key Findings. *https://crashstats.nhtsa.dot.gov/Api/Public/ViewPublication/812219*.

Nestler, E.J., Hope, B.T. & Widnell, K.L., 1993. Drug addiction: a model for the molecular basis of neural plasticity. *Neuron*, 11(6), pp.995–1006.

NIAAA, 2012. Immune Function genes, genetics, and the Neurobiology of Addiction. pp.1–8.

Nikodemova, M. & Watters, J.J., 2012. Efficient isolation of live microglia with preserved phenotypes from adult mouse brain. *Journal of Neuroinflammation*, 9(1), p.147.

Nilsen, N.J. et al., 2015. A role for the adaptor proteins TRAM and TRIF in toll-like receptor 2 signaling. *The Journal of biological chemistry*, 290(6), pp.3209–3222.

Nixon, Kimberly & Crews, F.T., 2002. Binge ethanol exposure decreases neurogenesis in adult rat hippocampus. *Journal of Neurochemistry*, 83(5), pp.1087–1093.

Noël, X. et al., 2007. Alcohol cues increase cognitive impulsivity in individuals with alcoholism. *Psychopharmacology*, 192(2), pp.291–298.

O'Callaghan, J.P., Sriram, K. & Miller, D.B., 2008. Defining "neuroinflammation". *Annals of the New York Academy of Sciences*, 1139(1), pp.318–330.

O'Neill, L.A.J. & Bowie, A.G., 2007. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nature Reviews Immunology*, 7(5), pp.353–364.

Okun, E. et al., 2010. TLR2 activation inhibits embryonic neural progenitor cell proliferation. *Journal of Neurochemistry*, 114(2), pp.462–474.

Olmos, G. & Lladó, J., 2014. Tumor necrosis factor alpha: a link between neuroinflammation and excitotoxicity. *Mediators of inflammation*, 2014(9), pp.861231–12.

Olofsson, P.S. et al., 2012. Rethinking inflammation: neural circuits in the regulation of immunity. *Immunological reviews*, 248(1), pp.188–204.

Olson, J.K. & Miller, S.D., 2004. Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. *The Journal of Immunology*, 173(6), pp.3916–3924.

Orre, M. et al., 2014. Acute isolation and transcriptome characterization of cortical astrocytes and microglia from young and aged mice. *Neurobiology of Aging*, 35(1), pp.1–14.

Oscar-Berman, M. & Marinković, K., 2007. Alcohol: effects on neurobehavioral functions and the brain. *Neuropsychology review*, 17(3), pp.239–257.

Osterndorff-Kahanek, E. et al., 2013. Gene Expression in Brain and Liver Produced by Three Different Regimens of Alcohol Consumption in Mice: Comparison with Immune Activation D.-S. Choi, ed. *PLoS ONE*, 8(3), pp.e59870–10.

Osterndorff-Kahanek, E.A. et al., 2015. Chronic ethanol exposure produces time- and brain region-dependent changes in gene coexpression networks. B. Bardoni, ed. *PLoS ONE*, 10(3), p.e0121522.

Paglinawan, R. et al., 2003. TGFbeta directs gene expression of activated microglia to an anti-inflammatory phenotype strongly focusing on chemokine genes and cell migratory genes. *Glia*, 44(3), pp.219–231.

Park, C. et al., 2005. TLR3-mediated signal induces proinflammatory cytokine and chemokine gene expression in astrocytes: Differential signaling mechanisms of TLR3-induced IP-10 and IL-8 gene expression. *Glia*, 53(3), pp.248–256.

Parkhurst, C.N. et al., 2013. Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor. *Cell*, 155(7), pp.1596–1609.

Pascual, M. et al., 2007. Intermittent ethanol exposure induces inflammatory brain damage and causes long-term behavioural alterations in adolescent rats. *European Journal of Neuroscience*, 25(2), pp.541–550.

Pascual, M.A. et al., 2011. Impact of TLR4 on behavioral and cognitive dysfunctions

associated with alcohol-induced neuroinflammatory damage. *Brain Behavior and Immunity*, 25(S1), pp.S80–S91.

Pascual-Lucas, M. et al., 2014. LPS or ethanol triggers clathrin- and rafts/caveolae-dependent endocytosis of TLR4 in cortical astrocytes. *Journal of Neurochemistry*, 129(3), pp.448–462.

Peltier, D.C. et al., 2010. Human neuronal cells possess functional cytoplasmic and TLR-mediated innate immune pathways influenced by phosphatidylinositol-3 kinase signaling. *Journal of immunology (Baltimore, Md. : 1950)*, 184(12), pp.7010–7021.

Piechota, M. et al., 2010. The dissection of transcriptional modules regulated by various drugs of abuse in the mouse striatum. *Genome biology*, 11(5), p.R48.

Pla, A., Pascual, M. & Guerri, C., 2016. Autophagy Constitutes a Protective Mechanism against Ethanol Toxicity in Mouse Astrocytes and Neurons. T. Amédée, ed. *PLoS ONE*, 11(4), p.e0153097.

Poltorak, A. et al., 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*, 282(5396), pp.2085–2088.

Préhaud, C. et al., 2005. Virus infection switches TLR-3-positive human neurons to become strong producers of beta interferon. *Journal of Virology*, 79(20), pp.12893–12904.

Probert, L., 2015. TNF and its receptors in the CNS: The essential, the desirable and the deleterious effects. *NEUROSCIENCE*, 302, pp.2–22.

Pruett, S.B. et al., 2004. Ethanol suppresses cytokine responses induced through Toll-like receptors as well as innate resistance to Escherichia coli in a mouse model for binge drinking. *Alcohol (Fayetteville, N.Y.)*, 33(2), pp.147–155.

Qi, J. et al., 2011. Painful pathways induced by TLR stimulation of dorsal root ganglion neurons. *Journal of immunology (Baltimore, Md. : 1950)*, 186(11), pp.6417–6426.

Qin, L. & Crews, F.T., 2012. Chronic ethanol increases systemic TLR3 agonist-induced neuroinflammation and neurodegeneration. *???*, 9(1), pp.1–1.

Qin, L. et al., 2008. Increased systemic and brain cytokine production and neuroinflammation by endotoxin following ethanol treatment. *Journal of Neuroinflammation*, 5(1), pp.10–17.

Qin, L. et al., 2013. NADPH oxidase and aging drive microglial activation, oxidative stress, and dopaminergic neurodegeneration following systemic LPS administration. *Glia*, 61(6), pp.855–868.

Qin, L. et al., 2007. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia*, 55(5), pp.453–462.

Ransohoff, R.M., 2016a. A polarizing question: do M1 and M2 microglia exist? *Nature Neuroscience*, 19(8), pp.987–991.

Ransohoff, R.M., 2016b. How neuroinflammation contributes to neurodegeneration. *Science*, 353(6301), pp.777–783.

Ransohoff, R.M. & Cardona, A.E., 2010. The myeloid cells of the central nervous system parenchyma. *Nature*, 468(7321), pp.253–262.

Reilly, S.M. et al., 2013. An inhibitor of the protein kinases TBK1 and IKK-ε improves obesity-related metabolic dysfunctions in mice. *Nature Medicine*, 19(3), pp.313–321.

Reinert, L.S. et al., 2012. TLR3 deficiency renders astrocytes permissive to herpes simplex virus infection and facilitates establishment of CNS infection in mice. *The Journal of clinical investigation*, 122(4), pp.1368–1376.

Robinson, G. et al., 2014. Neuroimmune pathways in alcohol consumption: evidence from behavioral and genetic studies in rodents and humans. *International review of neurobiology*, 118, pp.13–39.

Rolls, A. et al., 2007. Toll-like receptors modulate adult hippocampal neurogenesis. *Nature cell biology*, 9(9), pp.1081–1088.

Rosenberger, K. et al., 2014. The impact of single and pairwise Toll-like receptor activation on neuroinflammation and neurodegeneration. *Journal of Neuroinflammation*, 11(1), p.166.

Roussa, E., Bohlen und Halbach, von, O. & Krieglstein, K., 2009. TGF-beta in dopamine neuron development, maintenance and neuroprotection. *Advances in experimental medicine and biology*, 651, pp.81–90.

Roy, A. et al., 2006. Up-regulation of microglial CD11b expression by nitric oxide. *Journal of Biological Chemistry*, 281(21), pp.14971–14980.

Rubio, J.P. et al., 2008. Replication of KIAA0350, IL2RA, RPL5 and CD58 as multiple sclerosis susceptibility genes in Australians. *Genes and immunity*, 9(7), pp.624–630.

Rupprecht, R. et al., 2010. Translocator protein (18 kDa) (TSPO) as a therapeutic target for neurological and psychiatric disorders. *Nature reviews. Drug discovery*, 9(12), pp.971–988.

Sakharkar, A.J. et al., 2014. Effects of histone deacetylase inhibitors on amygdaloid

histone acetylation and neuropeptide Y expression: a role in anxiety-like and alcohol-drinking behaviours. *The international journal of neuropsychopharmacology*, 17(8), pp.1207–1220.

Salter, M.W. & Beggs, S., 2014. Sublime Microglia: Expanding Roles for the Guardians of the CNS. *Cell*, 158(1), pp.15–24.

Satoh, J.-I. et al., 2016. TMEM119 marks a subset of microglia in the human brain. *Neuropathology : official journal of the Japanese Society of Neuropathology*, 36(1), pp.39–49.

Schmid, C.D. et al., 2009. Differential gene expression in LPS/IFNgamma activated microglia and macrophages: in vitro versus in vivo. *Journal of Neurochemistry*, 109 Suppl 1(s1), pp.117–125.

Scumpia, P.O. et al., 2005. Double-stranded RNA signals antiviral and inflammatory programs and dysfunctional glutamate transport in TLR3-expressing astrocytes. *Glia*, 52(2), pp.153–162.

Sepulveda, F.E. et al., 2009. Critical role for asparagine endopeptidase in endocytic Toll-like receptor signaling in dendritic cells. *Immunity*, 31(5), pp.737–748.

Shannon, P. et al., 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome research*, 13(11), pp.2498–2504.

Shatz, C.J., 2009. MHC class I: an unexpected role in neuronal plasticity. *Neuron*, 64(1), pp.40–45.

Shemer, A. & Jung, S., 2015. Differential roles of resident microglia and infiltrating monocytes in murine CNS autoimmunity. *Seminars in immunopathology*, 37(6), pp.613–623.

Siddiqui, S.V. et al., 2008. Neuropsychology of prefrontal cortex. *Indian journal of psychiatry*, 50(3), pp.202–208.

Siegfried, A. et al., 2013. IFIT2 is an effector protein of type I IFN-mediated amplification of lipopolysaccharide (LPS)-induced TNF-α secretion and LPS-induced endotoxin shock. *Journal of immunology (Baltimore, Md. : 1950)*, 191(7), pp.3913–3921.

Skovira, J.W. et al., 2016. Cell cycle inhibition reduces inflammatory responses, neuronal loss, and cognitive deficits induced by hypobaria exposure following traumatic brain injury. *Journal of Neuroinflammation*, 13(1), p.299.

Smith, M.L. et al., 2016. Time-Course Analysis of Brain Regional Expression Network

Responses to Chronic Intermittent Ethanol and Withdrawal: Implications for Mechanisms Underlying Excessive Ethanol Consumption. A. M. Dopico, ed. *PLoS ONE*, 11(1), p.e0146257.

Sochocka, M., Diniz, B.S. & Leszek, J., 2016. Inflammatory Response in the CNS: Friend or Foe? *Molecular neurobiology*, 13(Suppl 1), pp.3391–19.

Starkman, B.G., Sakharkar, A.J. & Pandey, S.C., 2012. Epigenetics-beyond the genome in alcoholism. *Alcohol research : current reviews*, 34(3), pp.293–305.

Stefaniuk, M. et al., 2017. Matrix Metalloproteinase-9 and Synaptic Plasticity in the Central Amygdala in Control of Alcohol-Seeking Behavior. *Biological Psychiatry*, 0(0).

Stolyarova, A. et al., 2015. Reductions in frontocortical cytokine levels are associated with long-lasting alterations in reward valuation after methamphetamine. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, 40(5), pp.1234–1242.

Streit, W.J., Mrak, R.E. & Griffin, W.S.T., 2004. Microglia and neuroinflammation: a pathological perspective. *Journal of Neuroinflammation*, 1(1), p.14.

Substance Abuse and Mental Health Services Administration, 2012. Data Spotlight: More than 7 Milllion Children Live with a Parent with Alcohol Problems. *http://media.samhsa.gov/data/spotlight/Spot061ChildrenOfAlcoholics2012.pdf*.

Szabo, G. & Lippai, D., 2014. *Converging Actions of Alcohol on Liver and Brain Immune Signaling* 1st ed., Elsevier Inc.

Szabo, G. & Saha, B., 2015. Alcohol's Effect on Host Defense. *Alcohol research : current reviews*, 37(2), pp.159–170.

Szabo, G. et al., 2011. The unfolding web of innate immune dysregulation in alcoholic liver injury. *Alcoholism: Clinical and Experimental Research*, 35(5), pp.782–786.

Takamiya, R. et al., 2013. The interaction between Siglec-15 and tumor-associated sialyl-Tn antigen enhances TGF-β secretion from monocytes/macrophages through the DAP12-Syk pathway. *Glycobiology*, 23(2), pp.178–187.

Takeda, K. & Akira, S., 2004. TLR signaling pathways. *Seminars in Immunology*, 16(1), pp.3–9.

Takeda, K. & Akira, S., 2005. Toll-like receptors in innate immunity. *International immunology*, 17(1), pp.1–14.

Tang, S.-C. et al., 2007. Pivotal role for neuronal Toll-like receptors in ischemic brain injury and functional deficits. *Proceedings of the National Academy of Sciences*, 104(34), pp.13798–13803.

Tang, Y. & Le, W., 2016. Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases. *Molecular neurobiology*, 53(2), pp.1181–1194.

Tobar, N., Villar, V. & Santibanez, J.F., 2010. ROS-NFkappaB mediates TGF-beta1-induced expression of urokinase-type plasminogen activator, matrix metalloproteinase-9 and cell invasion. *Molecular and cellular biochemistry*, 340(1-2), pp.195–202.

Truitt, J.M. et al., 2016. Inhibition of IKKβ Reduces Ethanol Consumption in C57BL/6J Mice. *eNeuro*, 3(5), pp.ENEURO.0256–16.2016.

Uhlén, M. et al., 2015. Proteomics. Tissue-based map of the human proteome. *Science*, 347(6220), pp.1260419–1260419.

Uylings, H.B.M., Groenewegen, H.J. & Kolb, B., 2003. Do rats have a prefrontal cortex? *Behavioural Brain Research*, 146(1-2), pp.3–17.

Valero, J. et al., 2014. Long-term effects of an acute and systemic administration of LPS on adult neurogenesis and spatial memory. *Frontiers in Neuroscience*, 8(63), p.83.

van Schaarenburg, R.A. et al., 2016. The production and secretion of complement component C1q by human mast cells. *Molecular Immunology*, 78, pp.164–170.

Vengeliene, V. et al., 2009. Neuropharmacology of alcohol addiction. *British Journal of Pharmacology*, 154(2), pp.299–315.

Vetreno, R.P. & Crews, F.T., 2014. *Current hypotheses on the mechanisms of alcoholism* 1st ed., Elsevier B.V.

Vetreno, R.P., Qin, L. & Crews, F.T., 2013. Increased receptor for advanced glycation end product expression in the human alcoholic prefrontal cortex is linked to adolescent drinking. *Neurobiology of Disease*, 59(C), pp.52–62.

Villapol, S., T, T. & J, A., 2013. Role of TGF-β Signaling in Neurogenic Regions After Brain Injury. In *Trends in Cell Signaling Pathways in Neuronal Fate Decision*. InTech.

Vincent, C., Siddiqui, T.A. & Schlichter, L.C., 2012. Podosomes in migrating microglia: components and matrix degradation. *Journal of Neuroinflammation*, 9(1), p.190.

Vincenti, J.E. et al., 2016. Defining the Microglia Response during the Time Course of

Chronic Neurodegeneration B. Caughey, ed. *Journal of Virology*, 90(6), pp.3003–3017.

Weighardt, H. et al., 2004. Identification of a TLR4- and TRIF-dependent activation program of dendritic cells. *European Journal of Immunology*, 34(2), pp.558–564.

Werts, C. et al., 2001. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nature Immunology*, 2(4), pp.346–352.

Whitman, B.A. et al., 2013. The Cytokine mRNA Increase Induced by Withdrawal from Chronic Ethanol in the Sterile Environment of Brain is Mediated by CRF and HMGB1 Release. *Alcoholism: Clinical and Experimental Research*, 37(12), pp.2086–2097.

Wooten, R.M. et al., 2002. Toll-like receptor 2 is required for innate, but not acquired, host defense to Borrelia burgdorferi. *The Journal of Immunology*, 168(1), pp.348–355.

Wu, Y. et al., 2016. Induction of Siglec-1 by Endotoxin Tolerance Suppresses the Innate Immune Response by Promoting TGF-β1 Production. *The Journal of biological chemistry*, 291(23), pp.12370–12382.

Xie, N. et al., 2010. Glycogen synthase kinase-3 and p38 MAPK are required for opioid-induced microglia apoptosis. *Neuropharmacology*, 59(6), pp.444–451.

Xing, B., Bachstetter, A.D. & Van Eldik, L.J., 2011. Microglial p38α MAPK is critical for LPS-induced neuron degeneration, through a mechanism involving TNFα. *Molecular neurodegeneration*, 6(1), p.84.

Yang, X. et al., 1999. Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. *The EMBO journal*, 18(5), pp.1280–1291.

Yang, Y., Hill, J.W. & Rosenberg, G.A., 2011. Multiple roles of metalloproteinases in neurological disorders. *Progress in molecular biology and translational science*, 99, pp.241–263.

Yao, L. et al., 2013. Notch-1 signaling regulates microglia activation via NF-κB pathway after hypoxic exposure in vivo and in vitro. W. Hu, ed. *PLoS ONE*, 8(11), p.e78439.

Yoza, B.K., Hu, J.Y.-Q. & McCall, C.E., 2002. Inhibition of histone deacetylation enhances endotoxin-stimulated transcription but does not reverse endotoxin tolerance. *Journal of endotoxin research*, 8(2), pp.109–114.

Yu, L., Wang, L. & Chen, S., 2010. Endogenous toll-like receptor ligands and their

biological significance. *Journal of cellular and molecular medicine*, 14(11), pp.2592–2603.

Zhang, J. et al., 2014. Microglial CR3 activation triggers long-term synaptic depression in the hippocampus via NADPH oxidase. *Neuron*, 82(1), pp.195–207.

Zhang, Y. et al., 2014. An RNA-Sequencing Transcriptome and Splicing Database of Glia, Neurons, and Vascular Cells of the Cerebral Cortex. *Journal of Neuroscience*, 34(36), pp.11929–11947.

Zou, J. & Crews, F., 2010. Induction of Innate Immune Gene Expression Cascades in Brain Slice Cultures by Ethanol: Key Role of NF-κB and Proinflammatory Cytokines. *Alcoholism: Clinical and Experimental Research*, 34(5), pp.777–789.

Zou, J. & Crews, F.T., 2012. Inflammasome-IL-1β Signaling Mediates Ethanol Inhibition of Hippocampal Neurogenesis. *Frontiers in Neuroscience*, 6, p.77.

Zou, J.Y. & Crews, F.T., 2005a. TNF alpha potentiates glutamate neurotoxicity by inhibiting glutamate uptake in organotypic brain slice cultures: neuroprotection by NF kappa B inhibition. *Brain Research*, 1034(1-2), pp.11–24.

Zou, J.Y. & Crews, F.T., 2005b. TNF alpha potentiates glutamate neurotoxicity by inhibiting glutamate uptake in organotypic brain slice cultures: neuroprotection by NF kappa B inhibition. *Brain Research*, 1034(1-2), pp.11–24.

Zuloaga, D.G. et al., 2014. Sex differences in activation of the hypothalamic-pituitary-adrenal axis by methamphetamine. *Journal of Neurochemistry*, 129(3), pp.495–508.

Vita

Gizelle McCarthy (Robinson) was born in Massachusetts and grew up in the town of Hingham. After graduating from Hingham High School, she attended the University of Miami where she graduated with a B.S. in Biology. After graduating, Gizelle worked as a laboratory technician in the laboratory of Charles Stiles as the Dana-Farber Cancer Institute. In 2012, she began graduate school at the University of Texas in Austin and joined the laboratory of Adron Harris.

Permanent address (or email): gizellemmccarthy@gmail.com

This dissertation was typed by Gizelle McCarthy.